From the DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH

ENDOCRINE PROFILE IN FEMALE OLYMPIC ATHLETES -

OF IMPORTANCE FOR PHYSICAL PERFORMANCE AND IMPACT ON ANTI-DOPING TESTING

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Endocrine profile in female Olympic athletes- of importance for physical performance and impact on anti-doping testing

THESIS FOR DOCTORAL DEGREE (PhD)

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To Greta

"Tell me and I forget. Teach me and I remember. Involve me and I learn."

Benjamin Franklin

POPULAR SCIENCE SUMMARY OF THE THESIS

Anabolic hormones such as testosterone and growth hormone could be beneficial for physical performance. The second to fourth digit ratio, suggested as a measurement of testosterone exposure during fetal life, has also been related to athletic ability. There is however a lack of knowledge on how the body's own anabolic hormones and the digit ratio may affect muscle mass and physical performance in female elite athletes. In doping tests, the level of steroid hormones, for example testosterone, is measured in urine. In men, genetic variations have been shown to affect the steroid hormone levels. However, it is not known if genetic variations and hormonal contraceptives affect the steroid hormone levels in urine in female elite athletes.

The purpose of this thesis was to study the body's own anabolic hormones and the digit ratio in female Olympic athletes compared to untrained controls and to evaluate possible relationships with body composition and physical performance in the Olympic athletes. We also aimed to examine if genetic variations and hormonal contraceptives alter the steroid hormone levels in urine and if these hormone levels were different between athletes and controls.

We found relatively higher levels of anabolic hormones and a lower digit ratio (higher exposure to testosterone during fetal life) in the female Olympic athletes. The anabolic hormones were associated with increased muscle mass, stronger bone tissue and better physical performance. A low digit ratio was related to steroid hormone levels in urine but not to hormone levels in blood. The steroid hormone levels in urine were lower in the athletes than the controls and both genetic variations and hormonal contraceptives affected these hormone levels.

In conclusion, these findings reflect that the body's own anabolic hormones are important for muscle mass and physical performance in female Olympic athletes. This knowledge is valuable for the ongoing discussion about hyperandrogenism in female athletes. Based on the digit ratio hormonal exposure during fetal life appears to be associated with athletic performance. Furthermore, the association between the digit ratio and levels of steroid hormones in urine can be related to how these steroid hormones are processed in the body. Both genetic variations and hormonal contraceptives resulted in differences in steroid hormone levels in urine. The higher levels of these hormones in untrained women compared to Olympic athletes can be a result of increased androgen excretions by alternative elimination routes in the athletes.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Anabola hormoner såsom testosteron och tillväxthormon har uppbyggande effekter på muskelmassa och anses kunna öka den fysiska prestationsförmågan. Även fingerkvoten (längden på pekfingret delat med längden på ringfingret) som har lagts fram som mått på hormonmiljön under fosterlivet, har kopplats till prestation. Det saknas dock kunskap om hur kroppsegna anabola hormoner och fingerkvoten påverkar kroppssammansättning och fysisk prestation hos elitidrottande kvinnor. Vid dopingtest utvärderar man steroidhormoner, exempelvis testosteron, i urinprover. Tidigare studier på män har visat att det finns genetiska variationer som påverkar urinsteroidhormonnivåerna. Andra faktorer som kan påverka tolkningen av dopingtester är hormonella preventivmedel. Det är inte kartlagt hur genetiska variationer och hormonella preventivmedel påverkar urinsteroidprofilen hos kvinnliga elitidrottare.

Målet med denna avhandling var att studera kroppsegna anabola hormoner och fingerkvoten hos kvinnliga Olympiska idrottare och otränade kontroller, samt utvärdera eventuella kopplingar till kroppsammansättning och fysisk prestation hos kvinnliga Olympier. Vi hade även som syfte att studera om genetiska variationer och hormonella preventivmedel påverkar nivåerna av steroidhormon i urinen hos kvinnor och om dessa hormonnivåer skiljer sig åt mellan de kvinnliga Olympiska idrottarna och kontrollgruppen.

Vi fann högre nivåer av anabola hormoner och en lägre fingerkvot (mer påverkan av manliga könshormon under fosterlivet) hos de kvinnliga Olympiska atleterna. Dessa anabola hormoner var relaterade till ökad muskelmassa och ökad bentäthet samt bättre fysisk prestation. En lägre fingerkvot hade samband med högre nivåer av vissa steroidhormon i urin, men inte till hormonnivåer i blod. Nivåerna av steroidhormoner i urin var högre hos kontrollerna än de kvinnliga Olympiska atleterna och hos de sistnämnda var en högre träningsnivå relaterat till lägre urinsteroidhormonnivåer. Både genetiska variationer och hormonella preventivmedel påverkade urinsteroidhormonnivåer.

Sammanfattningsvis talar dessa resultat för att kroppsegna anabola hormoner spelar roll för ökad muskelmassa och fysisk prestation hos kvinnliga Olympiska idrottare. Denna kunskap är viktig för den pågående diskussionen om hyperandrogenism hos kvinnliga idrottare. Baserat på fingerkvoten, förefaller hormonmiljön under fosterstadiet ha samband med fysisk prestation och hormonnivåer i urin. Det senare talar för att fingerkvoten har en koppling till hur steroidhormoner omsätts i kroppen. Både genetiska variationer och hormonella preventivmedel påverkade urinsteroidprofilen hos kvinnor. Att otränade kontroller utsöndrar högre nivåer av androgener i urin kan hypotetiskt bero på att elitidrottskvinnor har ökad androgen utsöndring via alternativa utsöndringsvägar.

ABSTRACT

Endogenous androgens and insulin-like growth factor-I (IGF-I) are anabolic hormones that may contribute to increased muscle mass and possibly enhanced athletic performance. Furthermore, the second to fourth digit ratio (2D:4D ratio), suggested as an indirect measurement of prenatal androgen exposure, has been related to athletic ability. However, there is limited knowledge on the role of endogenous anabolic hormones for body composition and physical performance in female Olympic athletes. Moreover, the 2D:4D ratio has not been studied in relation to serum and urinary androgens and athletic performance in female elite athletes. In men, there are known genetic variations in genes coding for androgen metabolizing enzymes that affect the urinary androgen profile evaluated in doping tests. For women, less is known concerning the impact of genetic variations on the urinary androgens. In addition, the effects of hormonal contraceptive use on urinary steroid levels need to be investigated further.

The aim of this thesis was to evaluate the anabolic hormonal profile and the 2D:4D ratio in female Olympic athletes compared to untrained controls and in relation to body composition and physical performance. In addition, we aimed to investigate the relationship between the 2D:4D ratio and serum and urinary androgens. Furthermore, we studied the impact of hormonal contraceptives and genetic variations on the urinary steroid profile in female elite athletes and compared the urinary steroid between female athletes and controls.

In this cross-sectional study, including female Olympic athletes (n=106) and age- and BMI matched untrained controls (n=117), we found significantly higher precursor androgens dehydroepiandrosterone (DHEA), androstenediol (5-DIOL) as well as IGF-I, age-adjusted IGF-I and insulin-like growth factor binding protein-1 (IGFBP-1) in the athletes compared to controls. The 2D:4D ratio was significantly lower in the athletes indicating higher exposure to androgens during fetal life. The precursor androgens, IGF-I and IGFBP-1 were related to increased muscle mass and lower fat percentage. In the athletes, precursor androgens, dihydrotestosterone (DHT), IGF-I, IGFBP-1 and the 2D:4D ratio were associated with better performance in physical fitness tests. In addition, the 2D:4D ratio was associated with urinary androgens but not serum androgens. The controls excreted significantly higher concentrations of urinary androgen metabolites compared to the athletes, however serum androgens were comparable or higher among the athletes. For the athletes, training hours per week were negatively associated with some urinary androgens. Moreover, we found that genetic variations in UGT2B17 and CYP17A1 and hormonal contraceptive use had a significant impact on the urinary steroid profile in women.

In conclusion, endogenous anabolic hormones are associated with an anabolic body composition and enhanced physical performance in female Olympic athletes. Prenatal androgen exposure may also be of importance for athletic capacity in female Olympic athletes and possibly reflect androgen metabolism, since the 2D:4D ratio was associated with urinary androgen metabolite concentrations. Both genetic variations in UGT2B17, CYP17A1 and hormonal contraceptive use affects the urinary androgen levels in women. Our finding of higher urinary androgen concentrations in controls compared to the athletes and the negative association between training hours and urinary androgens is hypothetically due to increased androgen excretion via alternative excretion routes in female athletes.

LIST OF SCIENTIFIC PAPERS

I. Eklund E, Berglund B, Labrie F, Carlström K, Ekström L, Hirschberg AL. **Serum androgen profile and physical performance in women Olympic athletes**. *British journal of sports medicine*. 2017;51(17):1301-8.

II. Eklund E, Ekström L, Thörngren J-O, Ericsson M, Berglund B, Hirschberg AL. **Digit Ratio (2D:4D) and Physical Performance in Female Olympic Athletes**. *Frontiers in Endocrinology*. 2020;11(292):1-8.

III. Eklund E, Hellberg A, Berglund B, Brismar K, Hirschberg AL. **IGF-I and IGFBP-1 in relation to body composition and physical performance in female Olympic athletes**. 2021. *Submitted.*

IV. Schulze JJ*, Mullen JE*, Berglund Lindgren E, Ericsson M, Ekström L, Hirschberg AL. **The impact of genetics and hormonal contraceptives on the steroid profile in female athletes**. *Frontiers in Endocrinoly*. 2014;5(50):1-6.

V. Eklund E, Andersson A, Ekström L, Hirschberg AL. **Urinary steroid profile in elite female athletes in relation to serum androgens and in comparison with untrained controls.** 2021. *Submitted.*

*Shared first author.

CONTENTS

LIST OF ABBREVIATIONS

2D	Digit two
2D:4D ratio	Second to fourth digit ratio
3α-diol-3G	Androstane-3α, 17β-3 glucuronide
3α-diol-17G	Androstane-3α, 17β-17 glucuronide
4D	Digit four
5-DIOL	Androstenediol
5αAdiol-G	5α-androstane-3α, 17β-diol glucuronide
5βAdiol-G	5β- androstane-3α, 17β-diol glucuronide
A4	Androstenedione
A/Etio	Androsterone/etiocholanolone ratio
AAS	Anabolic androgenic steroids
ABP	Athlete biological passport
ACTH	Adrenocorticotropic hormone
ADT-G	Androsterone glucuronide
AR	Androgen receptor
BMD	Bone mineral density
CAS	Court of Arbitration of Sport
CMJ	Countermovement jump
COCs	Combined oral contraceptives
CRH	Corticotropic-releasing hormone
CYP	Cytochrome P450
DDR	Deutsche Demokratische Republic
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone- sulphate
DHT	Dihydrotestosterone
DSD	Disorders of sex development
DXA	Dual-energy X-ray absorptiometry
E2	Estradiol
EpiT	Epitestosterone
EpiT-G	Epitestosterone glucuronide

Etio-G	Etiocholanolone glucuronide
FAI	Free androgen index
FSH	Follicle-stimulating hormone
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
IGFBP-1	Insulin-like growth factor binding protein-1
IGF-I	Insulin-like growth factor-I
IGFSD	Insulin-like growth factor-I age-dependent reference range
IOC	International Olympic Committee
IRMS	Isotope ratio mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
NO	Nitric oxide
PCOS	Polycystic ovary syndrome
RIA	radioimmunoassay
Rt-PCR	Real-Time polymerase chain reaction
SHBG	Sex hormone-binding globulin
SJ	Squat jump
SOC	Swedish Olympic Committee
SULTs	Sulfotransferases
T	Testosterone
T/E	Testosterone/epitestosterone ratio
T-G	Testosterone glucuronide
TMS	Trimethylsilyl derivates
UGTs	Uridine-disphospho (UDP)-glucuronosyl transferases
WADA	World Anti-Doping Agency

1 PREFACE

Sport has been a part of society since the prehistoric age, for instance one of the most important sporting competitions today, the Olympic Games, originated in Greece around 3000 years ago. In 1900 women were allowed to compete in the Olympic games and since then the percentage of women participating has increased from 2.2 % to an expected 48.8 % in the Tokyo 2020 Games (1). As for endocrinology, diseases related to hormonal pathologies have been evident throughout history, however, the term hormone (from the Greek "excite") was introduced as late as 1905 (2) and it took until the 1940s before androgen synthesis was defined in women (3). With this in mind, it is not surprising that much remains to be clarified within the field of sports endocrinology for female athletes.

Having been born into a family that includes Olympic athletes, an Olympic gold medal winner, researchers, medical doctors and sports enthusiasts, one can wonder if it was nature or nurture that lead me to become an elite volleyball player and earn a medical degree. I took an early interest in sports endocrinology and when given the chance to start my PhD in Professor Angelica Lindén Hirschberg's group, I knew I was in the right place. The past six years of research have resulted in a thesis focusing on endogenous anabolic hormones relation to body composition and physical performance in female Olympic athletes. I also studied factors that may affect the urinary steroid profile and consequently be important for doping interpretation.

The research process has been a journey of both despair and success. I have suffered from classic writer's block and constantly questioned my research findings to the point of exhaustion. But through hard work and determination I have also won research awards and scholarships and been fortunate to present my research at congresses and in peer-reviewed journals. None of this would have been possible without committed supervisors, great colleagues and the support of the athletic community, family and friends. Hopefully, the results presented in this thesis will inspire future researchers and society in general in their effort to contribute and continue supporting research in sports endocrinology.

Emma Eklund, Stockholm 2021

2 INTRODUCTION

Androgens and growth factors such as testosterone (T) and insulin-like growth factor-I (IGF-I) are anabolic hormones, that may contribute to increased muscle mass and enhanced physical performance in athletes (4, 5). In men, the positive anabolic effects of endogenous androgens for physical performance are well established (4, 6). However, there is limited understanding of the role of endogenous anabolic hormones for body composition and physical performance in elite female athletes.

Over the last decade there has been an ongoing debate within the sporting community about the possible performance enhancing effect of endogenous anabolic hormones in the female athlete. Historically, in the majority of elite sports events, competitors have been divided according to gender due to the difference in physical performance between men and women (7). Recently circulating T was proposed as the most important factor explaining the sex difference in athletic performance (4). Therefore, in 2011, the International Associations of Athletics Federations, and in 2012 the International Olympic Committee (IOC) implemented regulations on female hyperandrogenism in sports related to rare disorders of sex development (DSD). In summary it was stated that female athletes with endogenous testosterone levels in the male range and functioning androgen receptors (AR) were not eligible to compete in female sports events. These rules were intended to ensure fairness in women's elite sporting competition and not to determine sex or gender in the athlete (4, 8). Supporters argued that circulating T levels, in the male range (approximately fifteen times higher), clearly imply an advantage for physical performance (4, 8-11). However, critics have highlighted a lack of scientific data supporting these claims as well as other possible factors that may explain the differences in physical performance (12-14). The current knowledge about the anabolic effects of T in women was mainly derived from animal studies, investigations including only men or case reports and the Deutsche Demokratische Republic (DDR) records of the effects of anabolic steroids (i.e. doping) in female athletes (4, 15). Athletes affected by these regulations appealed to the Court of Arbitration of Sport (CAS), resulting in the regulations being suspended in 2015 pending further scientific evidence of the role of endogenous anabolic hormones in athletic performance in women. Since then, new scientific evidence has been published and reviewed by CAS, resulting in updated regulations being adopted in 2018.

When starting this PhD in 2015 there were only two publications on endogenous T and physical performance in female athletes. One by Cardinal and Stone (16) including 22 female athletes,

reporting a positive relation between circulating T and counter-movement jump (CMJ) performance. The second by Crewther and Christian (17), found no correlation between T levels and performance, however this study only included 4 female athletes. In 2017, study I included in this thesis was published providing scientific results on the relation between endogenous androgens, body composition and physical performance in female Olympic athletes. More data demonstrating a positive effect by androgens on physical performance in women was later published (8, 18, 19). In an effort to explore other possible factors important for athletic performance in women we continued by investigating endogenous IGF-I and insulin-like growth factor binding protein-1 (IGFBP-1) (study III) and the second to fourth digit ratio (2D:4D ratio) (study II), and their relationships with physical performance and body composition in female Olympic athletes.

Another ever present issue in the context of sports is doping i.e. the use of prohibited performance enhancing drugs such as anabolic androgenic steroids (AAS) and growth factors. Since steroid doping is evaluated by measuring androgen metabolites in urine, it is important to understand the factors that affect endogenous androgen levels. In women, the interpretation of endogenous androgen levels is complex due to cyclic variations of androgens during the menstrual cycle, the influence of hormonal contraceptives and possibly the impact of genetic variations on androgen metabolism (20). There are known polymorphisms in genes coding for enzymes responsible for androgen transformation that affect the urinary steroid profile in men and possibly also serum and urinary androgen metabolite levels in women (21). Therefore, in study IV, we examined the impact of genetics and hormonal contraceptives on the urinary steroid profile in women, and in study V we compared the urinary steroid profile between female elite athletes and untrained controls and investigated the relationship between the urinary steroid profile and exercise.

3 LITERATURE REVIEW

3.1 ANDROGENS IN WOMEN

3.1.1 Androgen production

In women, *de novo* synthesis of androgens from cholesterol e.g. *de novo* steroidogenesis takes place in the ovary, adrenal gland and during pregnancy the placenta, resulting in biologically active androgens (T and dihydrotestosterone (DHT)) and precursor androgens

(dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEA-S), androstenedione (A4), androstenediol (5-DIOL)). Androgens are released into the circulation and bind to the AR in the target tissue, resulting in a physiological response. Active androgens can directly bind to the AR, whereas precursor androgens require conversion to T or DHT before binding to the receptor (22).

The ovarian and adrenal gland production of androgens are part of the hypothalamic-pituitary-gonadal (HPG) - axis and the hypothalamic-pituitary- adrenal (HPA) - axis, respectively (22). The hypothalamus produces the gonadotropin-releasing hormone (GnRH) that binds to the secretory cells in the anterior pituitary gland, stimulating the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn stimulate androgen production in the ovary (Figure 1 and 2) (23, 24).

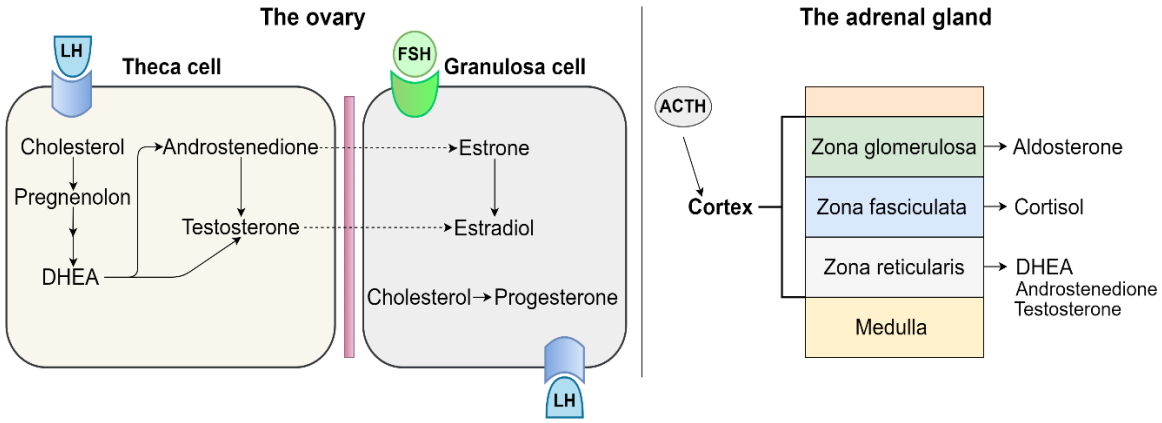

Figure 1: Androgen production of the ovary and adrenal gland. Androgens are synthesized in the theca cell by stimulation of LH and in the zona reticularis by stimulation of ACTH. In the ovary, androstenedione and testosterone can be released into the circulation or diffuse into the granulosa cell where FSH stimulates the transformation to estrone or estradiol via the enzyme aromatase. Prior to ovulation LH stimulates the granulosa cell to produce progesterone. ACTH=adrenocorticotropic hormone, DHEA=dehydroepiandrosterone, FSH=follicle-stimulating hormone, LH=luteinizing hormone. ©Emma Eklund.

The adrenal production of DHEA-S and DHEA as well as cortisol secretion is stimulated by adrenocorticotropic hormone (ACTH) from the pituitary gland, in turn stimulated by corticotropic-releasing hormone (CRH) from the hypothalamus (22) (Figure 1 and 2). Both the HPG and HPA-axis are regulated by negative feedback mainly by estrogens, progesterone and cortisol respectively, as well as by other endogenous hormones and environmental factors (22) (Figure 2).

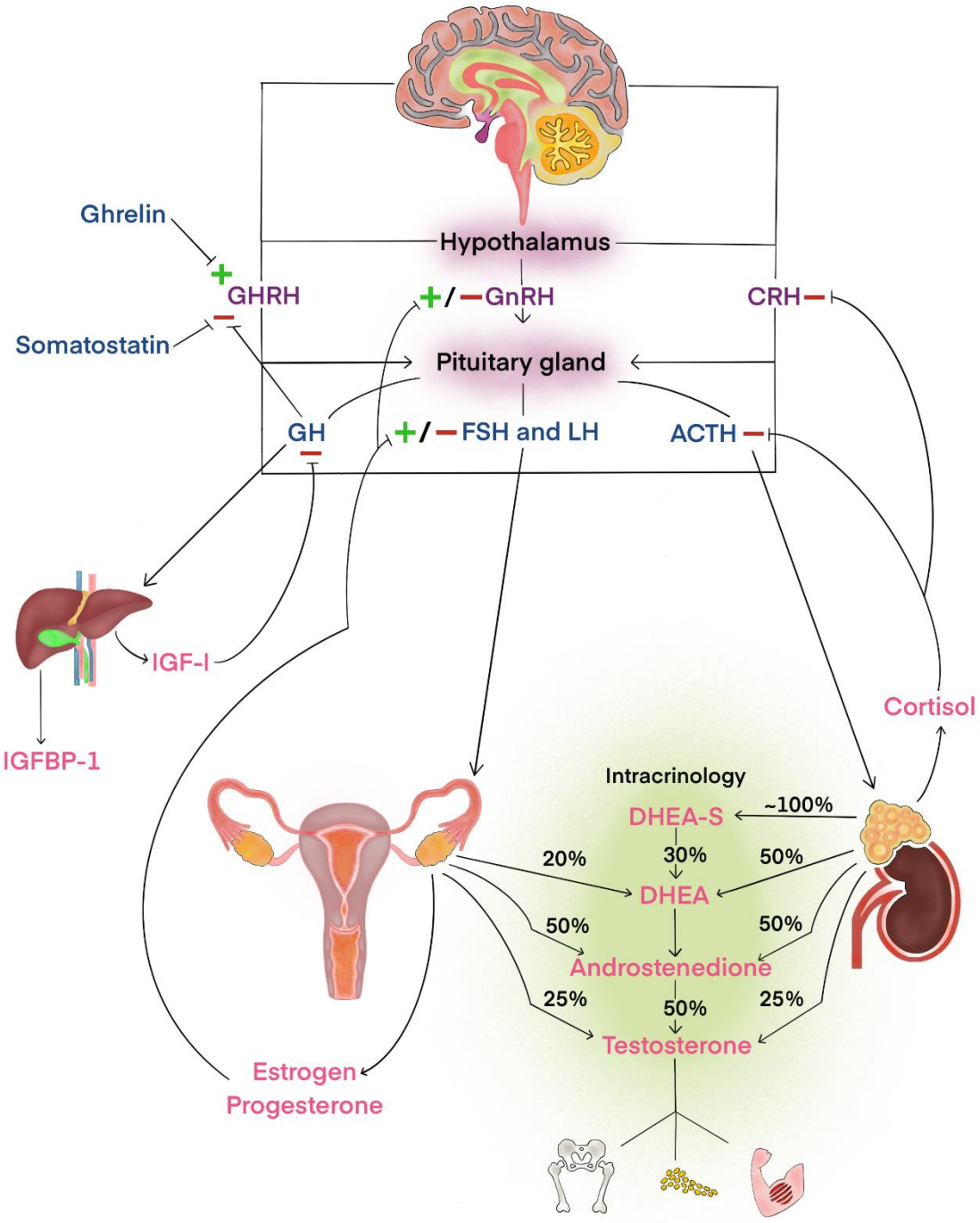

Figure 2: Illustration of the HPG-axis, HPA-axis and GH/IGF-I axis. The intracellular metabolism of precursor androgens by the mechanism of intracrinology and negative feedback mechanism of each hormonal axis are shown. The feedback of estrogen and progesterone on FSH and LH varies during the menstrual cycle. + indicates positive feedback, – indicates negative feedback. ACTH=adrenocorticotropic hormone, CRH=corticotrophin-releasing hormone, DHEA-S=dehydroepiandrosterone-sulphate, DHEA=dehydroepiandrosterone, FSH=follicle-stimulating hormone, GH=growth hormone, GHRH=growth hormone-releasing hormone, GnRH=gonadotropin-releasing hormone, IGF-I=insulin-like growth factor-I, IGFBP-1=insulin-like growth factor binding protein-1, LH=luteinizing hormone. ©Emma Eklund.

In premenopausal women, T is directly produced by the ovary (25%) and by the adrenal gland (25%) whereas the remaining major part is derived from precursor androgens (23, 25, 26). The biotransformation of precursor androgens to active androgens within the tissue-specific cell, referred to as intracrinology or intracrine androgen metabolism, is especially important in women (Figure 2 and 3) (22, 26). DHT is primarily derived by T conversion via the enzyme 5α-reductase, in the target tissue (25). These intracellular processes are possible in tissues that express steroid metabolizing enzymes such as in muscle, bone, skin and liver among others (22, 27).

3.1.2 Sex hormone-binding globulin

Both active androgens and precursor androgens derived from *de novo* steroidogenesis are transported in circulation to the target tissue, mainly by binding to sex hormone-binding globulin (SHBG) produced in the liver. Serum T binds strongly to SHBG (65-75%) and with less affinity to albumin (25-35%), whereas 1-3 % circulates as free T (20). Since only free active androgens are considered biologically active, SHBG levels in serum regulate androgen activity (20, 28). By calculating the free androgen index (FAI),$((T/SHBG) \times 100)$, free T can be estimated. Several factors affect SHBG levels, for example pregnancy and thyroid hormones increase SHBG, whereas high body weight, insulin and IGF-I decrease SHBG (29). In addition, hormonal contraceptives increase SHBG, where the elevation in SHBG levels is dependent on the estrogen dose and type and the type of progestin component (29, 30).

3.1.3 The androgen receptor

In the target tissue, the biological actions of androgens are mediated via the AR, a ligand-controlled transcription factor part of the nuclear receptor superfamily located in the nucleus of target cells. The AR is present in several tissues important for body composition and physical performance such as bone, skeletal muscle and adipose tissue (31). Binding of T or DHT to the AR initiates conformational changes allowing the AR to activate transcription of androgen target genes (24, 32). This process is influenced by co-regulators affecting the ligand sensitivity and DNA binding capacity of the AR (32). In the final step, after binding to the AR, active androgens are metabolized into androgen metabolites in the target cell, released into circulation and then excreted mainly in urine, but also in feces and sweat (33, 34).

3.1.4 Serum concentrations

In premenopausal women, serum androgen concentrations, in descending order are DHEA-S, DHEA, A4, T and DHT (22, 25). Serum concentrations of precursor androgens, active androgens and certain androgen metabolites are presented in table 1.

Table 1. Endogenous androgens in women, serum concentrations determined by LC-MS/MS.

Precursor Androgens	**LC-MS/MC**
DHEA-S	6.0 (3.4-9.6) µmol/L[A] 4.3 ± 0.02 µmol/L [B]
DHEA	7.1 (4.2-11.8) nmol/L[A] 3.4 ± 0.03 nmol/L[B]
A4	5.9 (3.3-9.2) nmol/L[A]
5-DIOL	1.5 ± 0.07 nmol/L[D]
Active androgens	
T	0.3 (0.2-0.5) nmol/L[A] 1.1 ± 0.09 nmol/L[B] 0.0-1.7 nmol/L[C]
DHT	0.4 ± 0.01 nmol/L[B]
Androgen metabolites	
ADT -G	89 ± 0.7 nmol/L[B] 2.1-170 nmol/L[D]
3α-diol- 3G	0.5-9.2 nmol/L[D]
3α-diol-17G	0.5-12 nmol/L[D]
Etio-G	-

[A] O'Reilly et al (2017). n=49 women, age 23-32 y. Median and interquartile range (35).
[B] Zang et al (2017). pooled commercial serum. Mean and standard deviation for female serum (36).
[C] Handelsman et al (2018). Summary of 9 research studies measuring serum T in healthy women, age <40 y. Lower-upper 95 % confidence interval (4).
[D] Labrie et al (2006). 47 premenopausal women. Age 30-35 y. 377 postmenopausal women. Age 55-65y min – max. Converted values nmol/L from Schiffer et al (2018) (27, 37).

3α-diol- 3G=androstane-3α,17β-3 glucuronide, 3α-diol-17G=androstane-3α,17β-17 glucuronide, 5-DIOL=androstenediol, A4=androstenedione, ADT-G=androsterone-glucuronide, DHEA-S=dehydroepiandrosterone-sulphate, DHEA=dehydroepiandrosterone, DHT=dihydrotestosterone, Etio-G=etiocholanolone-glucuronide, LC-MS/MS=liquid chromatography-tandem mass spectrometry, T=testosterone.

In women, the gonadal androgen productions starts during puberty, peaking at age 20-25 (4) whereas the adrenal androgen production is initiated during adrenarche (age 8 in girls) and reaches its peak at around 30-40 years of age (27). Several of the endogenous androgens decline with age and some vary across the normal menstrual cycle (23, 25, 31, 38). The physiological decline in DHEA-S, DHEA, T and A4 with age seems to be unrelated to natural menopause, whereas oophorectomy has been reported to reduce androgen levels by half (25, 38).

3.1.5 Importance of DHEA for women

DHEA and DHEA-S are the most abundant serum steroids in women. DHEA synthesis takes place in the adrenal gland (50%), in the ovaries (20%) and by conversion of DHEA-S (30%). Whereas, DHEA-S is predominantly produced in the adrenal glands (20, 23, 25, 26) (Figure 3). In women a major portion of active androgens is produced by transformation of precursor androgens, mainly DHEA, by the mechanism of intracrinology (33). Circulating DHEA diffuses into the cell and is locally transformed into active androgens (T and DHT) able to bind to the AR. By contrast, in the classic endocrine route active androgens secreted by the gonads and adrenal gland are transported in the circulation to the target tissue where they bind directly to the AR without the need for prior transformation (26, 33, 34). In the final step, active androgens are metabolized into mainly glucuronide metabolites and then released into the circulation. During this process very small amounts of active androgens escape from the cell, hence not altering T and DHT serum levels significantly (27) (Figure 3). It has been suggested that in women, androgen metabolites reflect androgen activity and indirect tissue exposure to androgens in a more reliable way compared to serum T alone (37). It is now possible to accurately analyze serum levels of precursor androgens, active androgens and androgen metabolites in women using mass spectrometry-based methods (39-41).

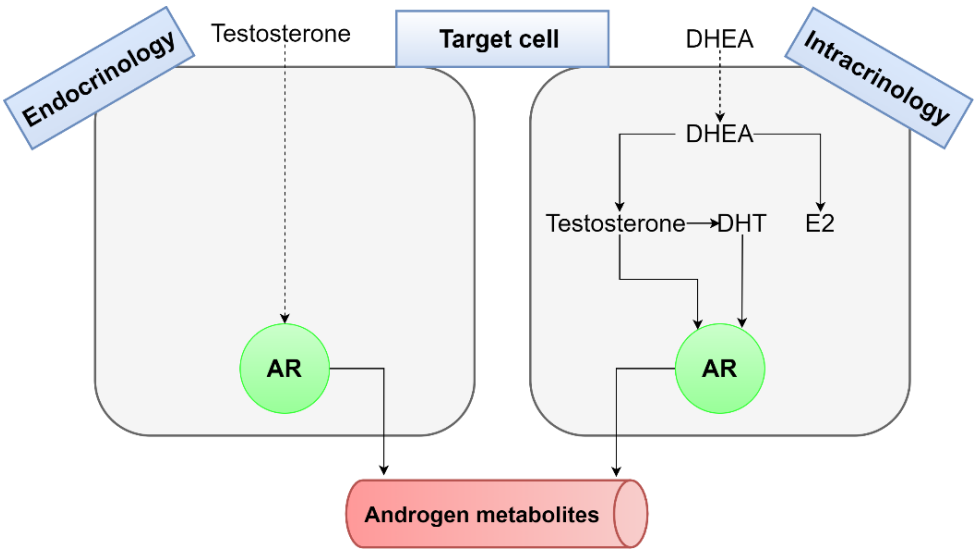

Figure 3: Schematic picture showing the classic endocrine route of testosterone and the intrecellular metabolism of DHEA. AR=androgen receptor, DHEA=dehydroepiandrosterone, DHT=dihydrotestosterone, E2=estradiol. ©Emma Eklund.

3.2 ANDROGEN METABOLISM

The androgen metabolism is complex, for clarity it can be divided into *de novo* steroidogenesis, intracrinology (also known as intracellular metabolism in peripheral androgen target tissue), see section 3.1.1, 3.1.5 (22, 26, 33), and finally inactivation of androgens by conjugation. The inactivation of androgens occurs in most tissues, although the liver is the major site. Conjugation of androgens makes the compound more water soluble, thereby facilitating excretion via mainly urine and bile (21, 22). In addition, feces and sweat have been suggested as alternative minor excretion routes although this remains to be studied in elite athletes (42-44).

3.2.1 Biotransformation of androgens

Enzymes from the cytochrome P450 (CYP) family initiate the first step of steroid synthesis. CYP17A1 is responsible for the 17-hydroxylation of pregnenolone and progesterone and formation of precursor androgens, DHEA and A4 (21, 22, 34). It has also been suggested that CYP17A1 is involved in the formation of epitestosterone (EpiT), an epimer of T important in the context of doping testing (21, 45). In the next step various hydroxysteroid dehydrogenases enzymes are involved in the formation of T from DHEA, A4 and 5-DIOL (34). The active androgen T can then be transformed by either 5α-reductase to the active form 5αDHT or by

5β-reductase to 5βDHT, the former process occurs mainly in the liver and is important to maintain androgen homeostasis (22). T and A4 can also be biotransformed into estrogens via the enzyme CYP19A1 (aromatase) (Figure 4) (21).

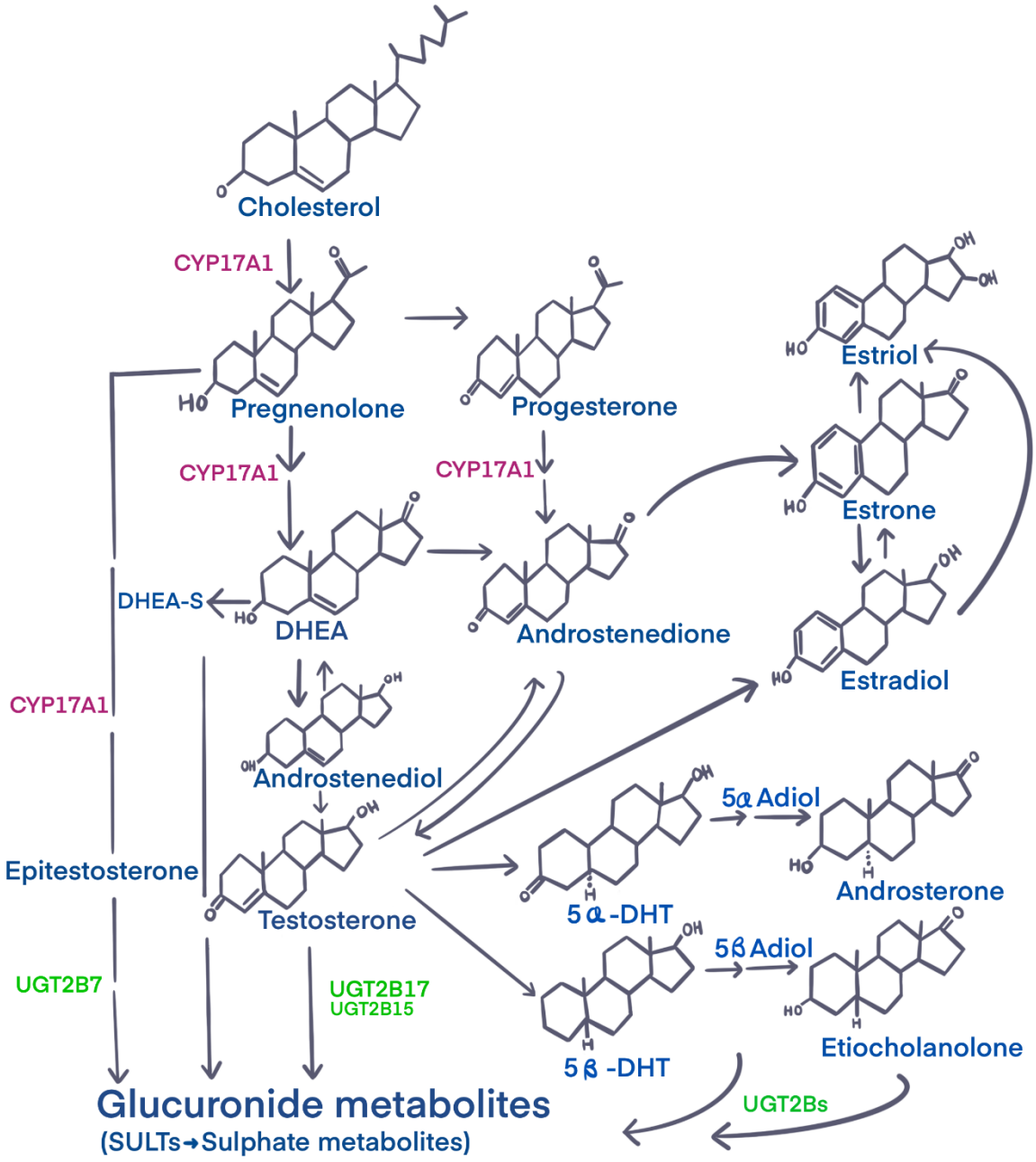

Figure 4: Illustration of the androgen metabolism. Important enzymes highlighted in purple and green, CYP17 and UGTs. 5αAdiol=5α-androstane-3α,17β-diol glucuronide, 5βAdiol=5β-androstane-3α,17β-diol glucuronide 5α-DHT=5α-dihydrotestosterone, 5β-DHT=5β-dihydrotestosterone, DHEA=dehydroepiandrosterone, DHEA-S=dehydroepiandrosterone sulphate, UGT2B=UDP glucuronosyl transferase 2B. For clarity, androstenedione=A4, androstenediol=5-DIOL. ©Emma Eklund.

5α-DHT can then be metabolized to androsterone glucuronide (ADT-G), androstane-3α, 17β-3 glucuronide (3α-diol-3G) and androstane-3α, 17β- 17 glucuronide (3α-diol-17G) via the 5α-reduction pathway, whereas 5β-DHT is converted to etiocholanolone glucuronide (Etio-G) via the 5β-reduction pathway (34, 46, 47).

The uridine-diphospho (UDP)-glucuronosyl transferases (UGTs) are the enzymes mainly responsible for the final formation of androgen metabolites, by glucuronidation, i.e. phase II metabolism (47, 48). The majority of androgens are metabolized in this manner, however small amounts are conjugated and excreted as sulphate metabolites after transformation by sulfotransferases (SULTs) (Figure 4) (21). These conjugated compounds are considered inactive, however while glucuronidation is irreversible, sulphate conjugation is reversible (22). After conjugation, androgen metabolites are excreted mainly in urine (21).

There are known polymorphisms in the genes coding for CYP17A1, UGTs and SULTs that affect the urinary androgen metabolite levels in men (45, 49-51). In women less is known about how genetic polymorphisms affect serum and urinary androgen metabolites levels.

3.2.1.1 CYPs

As previously mentioned, CYP 17A1 is responsible for the hydroxylation of pregnenolone and progesterone (Figure 4) (21, 22, 34) and has been suggested to be involved in the formation of EpiT (21, 45). A promotor region polymorphism (T>C exchange) in the CYP17A1 gene has been described, with the CC variant hypothesized to upregulate gene expression, possibly affecting steroid hormone levels (52). In men, this polymorphism has been associated with significantly higher excretion of epitestosterone glucuronide (EpiT-G) in urine (45).

3.2.1.2 UGT2B

UGTs are divided into UGT1 and UGT2, in which the latter is subdivided into UGT2A and UGT2B. In the UGT2B subfamily the enzymes UGT2B17, UGT2B15 and UGT2B7 are responsible for the androgen glucuronidation due to their high capacity to conjugate androgens (Figure 4) (47, 48). T is mainly metabolized by UGT2B17 and to a lesser extent by UGT2B15 and UGT2B7. UGT2B7 has been suggested as the main enzyme responsible for the glucuronidation of EpiT in vitro (21, 45). In men with a deletion polymorphism in the UGT2B17 gene (del/del), urinary testosterone glucuronide (T-G) levels were negligible and significantly lower compared to individuals with one (ins/del) or two copies (ins/ins) of UGT2B17 (21, 45, 49). For UGT2B7 and UGT2B15, polymorphisms have been identified that lead to a shift in amino acids. For UGT2B7 a missense polymorphism results in the replacement of histidine with tyrosine at position 268 (45, 53) and a polymorphism in the UGT2B15 gene

leads to a change of aspartate to tyrosine at position 85 (21, 45). In men, individuals with a UGT2B7 YY genotype demonstrated higher serum androgens compared to the HH and HY genotype, although urine levels of DHT, T and the testosterone/epitestosterone (T/E) ratio were not associated with the UGT2B7 polymorphism (54).

3.2.2 Doping detection

Some athletes use doping agents such as AAS, growth hormone (GH) and/or IGF-I to increase muscle growth and enhance performance (5, 55, 56). Currently, androgen abuse is evaluated using traditional testing and/or the athlete biological passport (ABP). Traditional testing refers to the analysis of androgen metabolites in urine in a single sample and calculation of specific ratios of urinary steroid, comparing these to population-based reference ranges, whereas the ABP implements a longitudinal approach.

To detect androgen abuse, the urinary steroid profile is currently determined using gas chromatography-tandem mass spectrometry analysis (GC-MS/MS), analyzing the urinary biomarkers T-G, EpiT-G, ADT-G, Etio-G, 5α-androstane-3α,17β-diol glucuronide (5αAdiol-G), 5β-androstane-3α,17β-diol glucuronide (5βAdiol-G). Since GC-MS/MS cannot distinguish between exogenous and endogenous androgens several ratios have been determined that indirectly measure androgen abuse, such as the T/E ratio. In men, exogenous T abuse increases urinary T-G and decreases its epimer EpiT-G resulting in an increased T/E ratio (57-59). In women, the effects of exogenous T on EpiT-G levels are more variable (60, 61). Traditionally, in elite athletes a T/E ratio above 4 is indicative of T abuse (21, 62). Additional ratios are also determined and atypical findings confirmed using the isotope ratio mass spectrometry (IRMS), able to distinguish between endogenous and exogenous androgens (58, 59).

The ABP consists of the hematological module introduced in 2009, followed by the steroid module initiated in 2014. The steroid module evaluates urinary androgens, however serum androgen levels may be added in the future (63). Furthermore, the endocrine module is currently under development focusing on the detection of GH, IGF-I and insulin doping (57, 58). The ABP monitors selected biomarkers using a longitudinal approach. Individual reference limits for these biomarkers can be calculated using specific algorithms and a Bayesian statistical method is used to progressively shift the comparison of the athlete's androgen levels from population-based reference ranges to the athlete's own reference ranges (59). Any significant change in these individual reference ranges raises suspicions of doping. By monitoring the individual athletes over time, subtle changes in biomarkers can be detected. In

addition, confounding factors such as genetic polymorphisms can be added to the statistical model and accounted for (62).

Both endogenous and exogenous factors can affect the interpretation of the urinary steroid profile. Genetic polymorphisms are the major confounders (21, 62), which could lead to both false negative and false atypical findings (21, 49, 64). The implementation of the ABP increases the chance to detect doping in men (65, 66). However, in women T administration is not necessarily detected either by traditional testing or the ABP (61, 67). Suggested important confounding factors with regard to doping detection in women are hormonal fluctuations during the menstrual cycle and the use of hormonal contraceptives (62, 68-70). Prior to study IV, part of this thesis, only one small report (n= 4 women) investigating the effects of hormonal contraceptives on the urinary steroid profile had been published. The authors found that hormonal contraceptives affect the T/E ratio, suggesting suppression of EpiT-G as a possible mechanism responsible for this change (68, 71).

3.3 PRENATAL ANDROGEN EXPOSURE

During pregnancy, the fetal/placental endocrine system produces and metabolizes steroid hormones in complex interaction with the maternal hormonal system (72). Androgens in prenatal blood/fetal blood can be determined directly by cordocentesis during the second or third trimester. However, due to the increased risk to the fetus, this procedure is rarely implemented and then only in high-risk pregnancies. Fetal androgens can also be determined at birth from umbilical cord blood, then reflecting only late gestation. Indirect approaches to measure fetal androgens include amniocentesis (restricted to high-risk pregnancies) during the second or third trimester or maternal blood sampling to evaluate hormonal levels (72, 73). Due to the invasive nature of these procedures and the risk to the pregnancy/fetus other indirect measurements of the prenatal androgens have been proposed such as the 2D:4D ratio i.e. the length of the second digit divided by the fourth digit (Figure 5) (74).

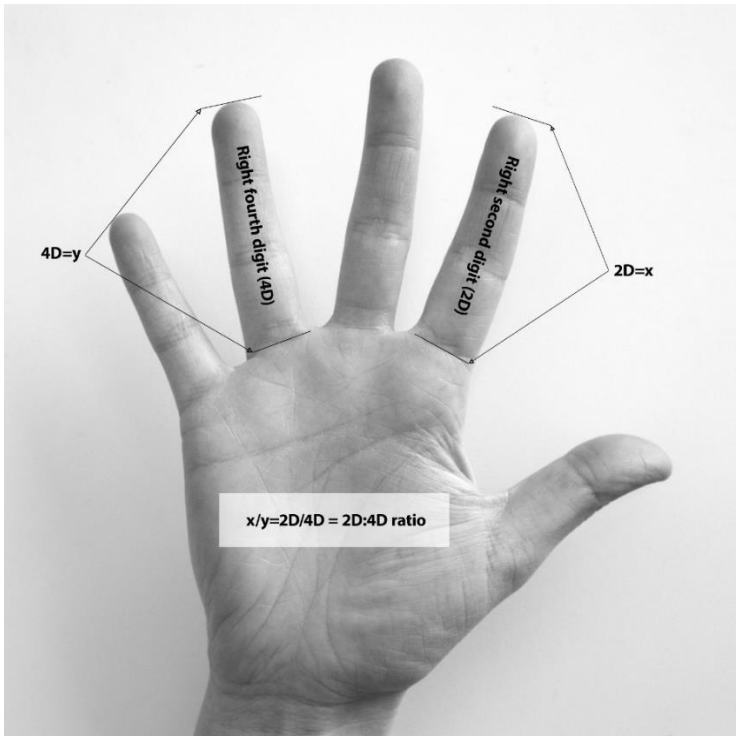

Figure 5: Measurement and calculation of the second to fourth digit (2D:4D) ratio. Photo: Emma Eklund. Originally published and reprinted from Frontiers in Endocrinology, vol 11, article 292, p 1-8; Copyright 2020. Open access. CC-BY license (75).

3.3.1 The 2D:4D ratio

Prenatal testosterone and estrogen levels have been suggested to influence the formation of the 2D:4D ratio (74, 76, 77). Previous studies have reported that the 2D:4D ratio is set during the first trimester of fetal development, as early as 9 weeks of gestation, with high levels of androgens during fetal life resulting in a low 2D:4D ratio (74, 78). It is considered a sexually dimorphic trait (74, 79) and does not change substantially with age in adults (74, 80).

In light of this, researchers have suggested the 2D:4D ratio may be an indirect measurement of prenatal androgen exposure (74). However, it should be noted that this concept has recently been debated (81-84). In table 2, studies examining the relationship between 2D:4D ratio and prenatal androgen exposure are summarized (73, 76-78, 85-88).

Table 2: Studies investigating prenatal androgen exposure and 2D:4D ratio.

Author (citation)	Method	Results
Lutchmaya 2004 (76)	FT and FE in amniotic fluid, second trimester. 2D:4D r at follow-up, child age 2 y. n=33 children (15 f, 18 m)	FT/FE (high FT in relation to FE) significant negative association with 2D:4D r.
Ventura 2013 (85)	FT in amniotic fluid and T maternal blood sample. 2D:4D r in healthy born infants and mothers (n=106)	FT higher in males than in females. No correlation between FT and maternal T. FT in females negatively correlated with 2D:4D r.
Hickey 2010 (73)	Androgens in cord blood, androgens in maternal blood second and third trimester. 2D:4D r in offspring, adolescent girls (n=244) at measurement.	No significant correlations were found.
Hollier 2015 (86)	T, A4, DHEA, and estrogens in cord blood*. 2D:4D r in f and m offspring at 19-22 y (n=341).	Males lower 2D:4D r compared to females. No significant correlations between 2D:4D r and fetal androgens and estrogens at late gestation.
Whitehouse 2015 (87)	T in cord blood*. 2D:4D r in f and m offspring at 21-24 y (n=183).	Males lower 2D:4D r compared to females. No correlation significant correlations found between cord T and 2D:4D r.
Mitsui (2016) (88)	Adrenal androgens in cord blood*. 2D:4D r in f and m offspring at school-age (n=190)	Males lower 2D:4D r compared to females. DHEA negatively correlated with 2D:4D r in males.
Malas (2006), (78)	n= 161 aborted fetuses (78 f, 83 m, age 10-40 weeks). 2D:4D r established.	Significant difference in 2D:4D r between sexes. No significant change in 2D:4D r over gestational ages.
Zheng (2011) (77)	58 CD-1 mice (30 m, 28 f). Hind limb 2D:4D r measured. ER and AR estimate in 2D and 4D.	Sexually dimorphic trait in mice. Higher AR and ER in 4D than 2D. Inactivation/Activation of receptors influence the 2D:4D r.

2D:4D r=second to fourth digit ratio right hand, A4=androstenedione, AR=androgen receptor, ER=estrogen receptor, DHEA=dehydroepiandrosterone, f=female, FE=fetal estrogen, FT=fetal testosterone, m=male, T=testosterone, y=years old. *analyzed by liquid chromatography- tandem mass spectrometry (LC/MS/MS).

Previous investigations have reported that females with congenital adrenal hyperplasia, a condition with increased androgen exposure in utero, have a lower 2D:4D ratio compared to healthy controls (79), a recent meta-analysis revealed similar, but less strong findings (89). Additionally, women with complete androgen insensitivity syndrome, having no response to androgens due to a non-functioning AR, have higher 2D:4D ratios than control women (90, 91). The 2D:4D ratio has also been related to athletic ability and physical fitness (92, 93).

3.3.2 The 2D:4D ratio in sports

The 2D:4D ratio is considered lower in athletes compared to non-athletes. However, previous studies have focused on male athletes participating in power/explosive sports (94-96), whereas data on 2D:4D ratio in female top elite athletes is limited. One study examined the 2D:4D ratio in female varsity athletes and controls and found lower ratios in the athlete population (97). In agreement, a lower 2D:4D ratio has been found among female tennis, handball and fencing athletes when compared to nonathletic controls (98-100). These studies have included few participants and no female Olympic level athletes have been represented. Others have found an association between a lower 2D:4D ratio and sporting level or performance in specific sport such as alpine skiing, fencing and rowing (101-103).

The cause of the correlation between 2D:4D ratio and physical performance and the reason for the lower 2D:4D ratio in athletes compared to non-athletes are not known. Initially researchers debated a possible link to adult serum T levels, however, two meta-analyses from 2007 and 2019 concluded that the digit ratio is not related to adult serum T levels (104, 105). Others have proposed a connection between the 2D:4D ratio and genetic variations in the AR, although recent investigations have indicated no such association (104, 106). Furthermore, genetic variants in selected genes coding for certain androgen metabolizing enzymes (aromatase, 5a-reductase) and SHBG have been examined in relation to the 2D:4D ratio without significant results (104). However, no previous studies have examined potential associations between the 2D:4D ratio and serum and urinary androgen metabolites, more representative of phase II androgen metabolism, in women Olympic athletes.

3.4 GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR-I

The peptide hormone IGF-I is an anabolic hormone, stimulating protein synthesis and cell proliferation with positive effects on adult bone metabolism and skeletal muscle (107, 108). GH secreted in a pulsatile manner from the pituitary gland stimulates the production of IGF-I

from the liver and non-hepatic tissues. The stimulatory effect of GH is dependent on the presence of insulin and amino acids. During malnutrition GH cannot stimulate IGF secretion. The secretion of GH is regulated primarily by growth hormone-releasing hormone (GHRH) (stimulating), ghrelin (stimulating) and somatostatin (inhibiting) from the hypothalamus. Furthermore, free IGF-I regulates GH secretion by negative feedback (Figure 2) (108). The majority (98-99%) of circulating IGF-I is bound to IGF binding proteins (IGFBPs) (109), thus in serum we determine total IGF levels not free IGF-1. IGF-I binds to the transmembrane tyrosine kinase IGF-I receptor part of the tyrosine kinase receptor family. It also binds with lower affinity to the insulin receptor and the hybrid insulin/IGF receptor (109).

The anabolic effects of GH are to a large extent mediated by circulating IGF-I (55). In addition, locally produced IGF-I can act via para- and autocrine mechanisms (108-110). In contrast to GH, total IGF-I levels are more stable and show little day-to-day intraindividual variability (108). Furthermore, total IGF-I levels have been shown to be comparable between men and women (111). However, it is well known that total IGF-I levels decrease with age (112, 113). Steroid hormones (androgens and estrogens) are suggested to regulate the actions of GH and IGF-I (114). Androgens stimulate GH secretion and circulating IGF-I, after aromatization to estrogen (115). In women, the use of estrogen therapies, may alter the GH/IGF axis, with oral estrogens inhibiting hepatic IGF-I production by first-pass estrogen effects while non-oral administration has no effect or increases IGF-I (116). Furthermore, small to moderate changes in IGF-I levels have been reported during the menstrual cycle (117-120).

3.4.1 IGFBPs

The IGFBPs have serum and tissue specific expression and act as transport proteins, regulate IGF-I bioavailability and prolong the half-life of IGF-I (107, 109, 121). Currently, there are six known IGFBPs (109). Circulating IGF-I is primarily bound (approximately 75 %) in a ternary complex with IGFBP-3 or 5 and Acid-label unit, 20-25% is bound in a binary complex with other IGFBPs e.g. IGFBP-1 or IGFBP-2, whereas only approximately 1-2 % circulate in free form (5, 121). Only IGF-I in its free form or as a binary complex can diffuse from circulation to the interstitial fluid then being able to bind to the IGF-receptor (121). It is now recognized that IGFBPs also have IGF-I independent effects at the cellular level by binding to an integrin receptor (107, 109, 121, 122).

IGFBP-1 production takes place in the liver and is mainly regulated by insulin at transcriptional level and to a lesser extent by catecholamines, glucagon and cortisol. Insulin inhibits, whereas catecholamines, glucagon and cortisol stimulate the hepatic production of IGFBP-1 (109, 122).

Therefore, in the non-fed state when insulin levels are low, inhibition of IGFBP-1 decreases, resulting in higher IGFBP-1 levels, the effect being further augmented by glucagon and cortisol. In the well-fed state the opposite occurs resulting in lower IGFBP-1 levels and increased IGF-I bioavailability. Due to its strong relation to insulin, circulating IGFBP-1 is positively associated with insulin sensitivity and has therefore been proposed as a marker of the latter (109). Furthermore, hypoxia stimulates IGFBP-1 synthesis via hypoxia inducible factor 1-alpha (HIF-1-a) at transcriptional level (123, 124). IGFBP-1 stimulates vascular nitric oxide (NO) synthesis and thus vasodilatation and blood flow (122). High-intensity exercise is associated with hypoxic reactions and increased HIF-1a (125) and increased IGFBP-1 (110).

3.5 THE FEMALE ATHLETE

In women, endogenous hormones fluctuate during the menstrual cycle and can be affected by hormonal contraceptive use. Furthermore, endocrine disturbances are common in female elite athletes often resulting in menstrual dysfunction. These hormonal disturbances can be a result of intense training, energy deficiency or essential hyperandrogenism (8, 126). The latter is suggested to have anabolic effects on body composition and possibly performance enhancing effects of relevance for the female athlete.

3.5.1 The menstrual cycle

The normal menstrual cycle is approximately 22-34 days and is divided into the follicular-, ovulation- and luteal phase related to ovarian changes, or the secretory- and proliferative phase of the endometrium. During the normal menstrual cycle, gonadotropins and steroid hormones vary. The fluctuations in gonadotropins, estradiol (E2) and progesterone are well established (127-129). DHT levels are low in women and do not fluctuate during the menstrual cycle, whereas T levels increase simultaneously in response to the LH peak during ovulation and last for approximately 2-3 days (128-130). This mid-cycle increase in T is however small and is within the physiological range of premenopausal women (4). Possible cyclic variations in adrenal androgens are less studied, however several investigations have demonstrated no significant difference in 5-DIOL, DHEA and DHEAS levels across the menstrual cycle (129-131) (Figure 6). As for IGF-I, variations during the menstrual cycle have been described to be non-existent to modest, whereas IGFBP-1 levels show no change (117-120).

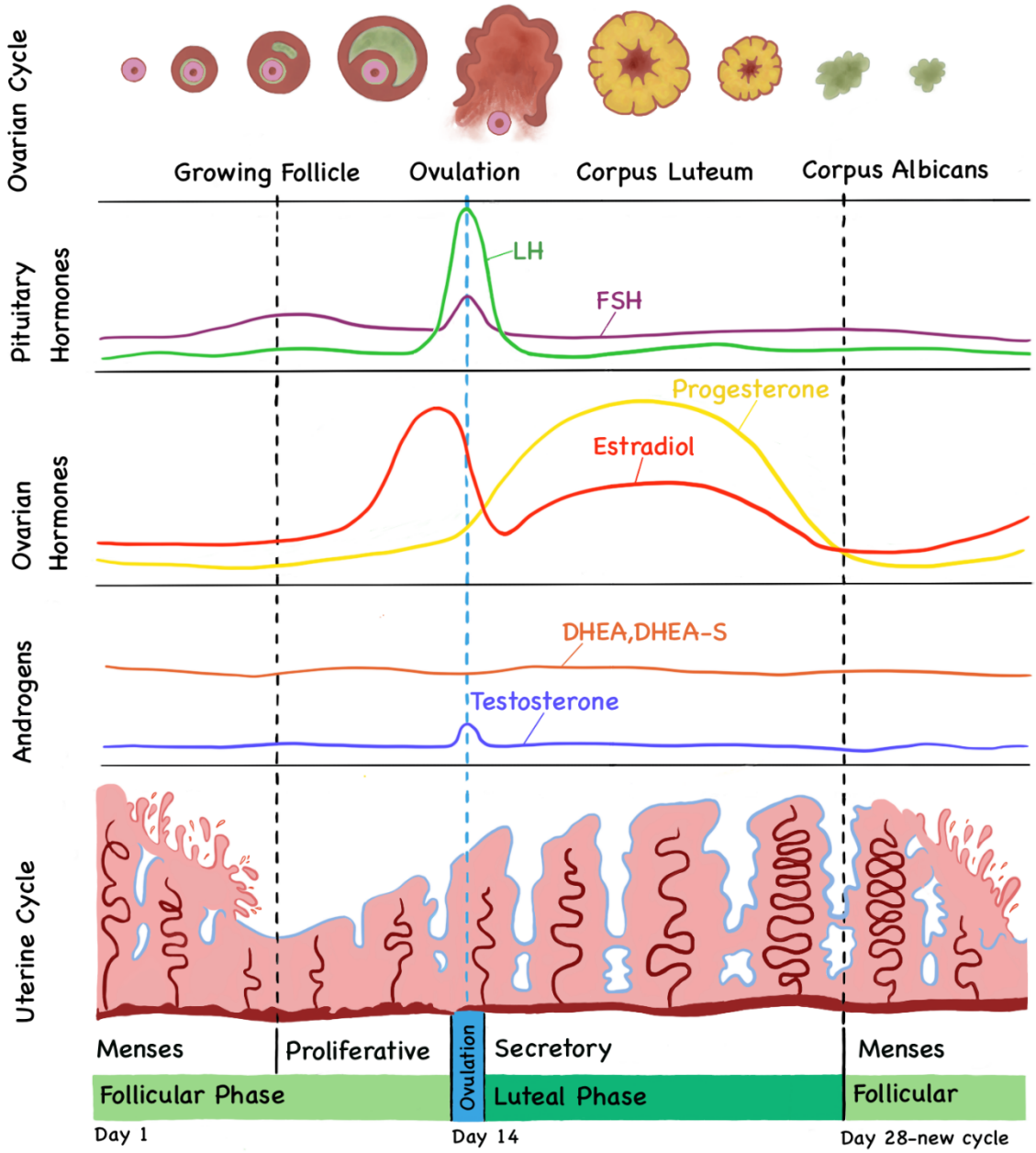

Figure 6: Illustration of the menstrual cycle showing hormone fluctuations during the different cycle phases. DHEA=dehydroepiandrosterone, DHEA-S=dehydroepiandrosterone-sulphate, FSH=follicle-stimulating hormone, LH=luteinizing hormone. ©Emma Eklund.

3.5.1.1 Menstrual dysfunction

Endocrine disturbances can result in menstrual dysfunction being categorized as amenorrhea (absence of menstruation during at least three months) or oligomenorrhea (5-9 periods during the past year occurring at intervals greater than six weeks). In the general population menstrual dysfunction ranges between 2-4 % (132, 133). Whereas, in female athletes it is frequent, ranging between 6-78%, the prevalence being higher among female athletes participating in endurance or esthetic sports (134-136).

Athletic amenorrhea, a form of functional hypothalamic amenorrhea is probably the most common cause of menstrual dysfunction among female athletes. Intense physical training and/ or low energy intake cause insufficient energy availability, leading to inhibition of the HPG axis. The disruption of the pulsatile release of GnRH results in reduced FSH and LH secretion and in turn lower estrogen, testosterone and progesterone, causing anovulation and amenorrhea (8, 137). In addition, insulin and IGF-I levels decrease whereas cortisol and IGFBP-1 are high. Taken together these hormonal changes can result in bone loss and increased risk of injury (8, 126).

An alternative explanation for menstrual dysfunction in female athletes is polycystic ovary syndrome (PCOS) (136), clinically characterized by polycystic ovaries and disturbed ovulation resulting in menstrual dysfunction, hirsutism and acne (8, 137). These symptoms are a results of increased androgen production in the ovaries, being attenuated by the disturbed negative feedback of GnRH resulting in higher LH. Furthermore, insulin resistance is common among PCOS women and insulin directly stimulates androgen production in the ovary and inhibits the hepatic SHBG production resulting in higher levels of free androgens able to bind to the AR (8). In contrast to functional hypothalamic amenorrhea, PCOS is associated with a more anabolic body composition and positive effects on physical performance (136, 138).

3.5.2 Hormonal contraceptives

Hormonal contraceptives include oral pills, patches, vaginal ring, subdermal implants, injections and intrauterine devices containing components of estrogen and/or progestin. The most common estrogen component in combined hormonal contraceptives is synthetic ethinylestradiol. Furthermore, the progestin component varies, as well as the dose of each component. The contraceptive effect is mainly induced by preventing ovulation by feedback on the HPG axis, alternatively altering the cervical mucus or by effects on the endometrium (139). In a previous study including Swedish female Olympic athletes approximately 40-50 %, used hormonal contraceptives (136).

3.5.2.1 Androgens

The anovulatory action of hormonal contraceptives is established via inhibition of the HPG axis, resulting in suppression of GnRH, followed by a decrease in gonadotrophins, ovarian steroid hormones and probably adrenal androgens. Combined oral contraceptives (COCs) induce a decrease in serum total T and increase in SHBG resulting in lower levels of free T (30, 139). An average decrease of 31 % in total T levels has been reported whereas the increase in SHBG is dependent on the estrogen and progestin component of the COC (30). The effects

on adrenal androgen levels are less established although recent findings show a decrease in these hormones (30, 140, 141). Women using hormonal contraceptives have significantly lower serum androgens and gonadotrophins and significantly higher levels of SHBG (142, 143), however changes in urinary androgen glucuronide metabolites are not fully elucidated.

3.5.2.2 GH/IGF-I

The effects of hormonal contraceptives on the GH/IGF-I system are dependent on the type of estrogen, the duration of treatment, dose and especially the route of administration. Although there is limited knowledge of the effect of hormonal contraceptives on IGF-I levels in premenopausal women, there is evidence that oral estrogens decrease circulatory IGF-I levels through hepatic first- pass effects. It has been suggested that estrogen metabolism in the liver suppresses hepatic IGF-I production and alters hepatic IGFBP production resulting in decreased IGF-I bioavailability. In contrast, non-orally administered estrogens result in less or no change in IGF-I levels. Further complicating the situation, combined treatment with a progestin can modulate the GH/IGF-I response depending on the androgenic profile of the progestin (116). Two previous investigations including female athletes found no significant difference in IGF-I levels depending on hormonal contraceptive use (143, 144). Whereas IGFBP-1 levels are higher in hormonal contraceptive users (136, 143).

3.5.3 Hyperandrogenism

Essential hyperandrogenism may be mild or severe depending on the cause. Hyperandrogenism is overrepresented among female athletes compared to women in general, and it is suggested that the higher endogenous androgen levels may results in performance enhancing effects (4, 136, 142). PCOS is the most common cause of mild hyperandrogenism, affecting approximately 6-10% of women. Women with PCOS often exhibit androgen levels in the upper normal female range and far from the male range (4). Previous studies have shown that women with PCOS have greater muscle strength compared to non-PCOS women which has been suggested to be related to the elevated androgen levels (145). Furthermore, female athletes with PCOS demonstrate higher aerobic performance values than non-PCOS athletes (138) and high BMD and lean mass (136).

Other rare conditions known as 46 XY DSD, result in significantly elevated active androgen levels, comparable to those of men (11). DSDs are congenital conditions leading to atypical chromosomal, gonadal or anatomical sex. There are several forms of 46 XY DSD, all exhibiting somewhat different phenotypes and various degree of androgen sensitivity. However, it is important to acknowledge that only 46 XY DSD individuals with a functioning AR can benefit

from the anabolic effects of T and DHT (146). Among female athletes, there is an overrepresentation of 46 XY DSD individuals, being 140 times more common compared to the general population (142). A recent study found that female athletes with 46 XY DSD had a median T level of 18 nmol/L, being significantly elevated compared to female athletes in general (median T 0.69 nmol/L) (142). Individual case studies have reported that testosterone lowering treatment in these individuals also reduced physical performance by approximately 5.7 % (147). Other conditions that may result in increased androgen levels are for instance androgen secreting tumors and AAS doping. Furthermore, both male and female transgender athletes may have high androgen levels depending on treatment (4).

3.6 ANABOLIC EFFECTS OF ANDROGENS

Androgens have anabolic effects on bone and muscle tissue, stimulate erythropoiesis and the immune system, and may affect behavior (4, 148, 149). It is well known that endogenous androgens and estrogens play an important role in bone health. T can bind to the AR expressed in both osteoblasts and osteocytes or act indirectly by aromatization to estrogen, both pathways promoting bone formation (149-151). In muscle, T increases the number of myonuclei and satellite cells and muscle fiber size and number. Furthermore, T increases erythropoietin levels resulting in higher hemoglobin concentrations (4). Taken together these effects are likely to be beneficial for athletic performance by a likely increase in muscle strength, bone formation and maximal oxygen uptake/ aerobic capacity.

In men, there is a well-established relationship between both exogenous and endogenous androgens and a more anabolic body composition and increased physical performance (4, 6, 16, 152-154). However, in women, especially female athletes, less is known about exogenous and endogenous androgen effects on body composition and physical performance.

3.6.1 Exogenous androgens, body composition and physical performance

In young healthy males, T administration follows a dose-response relationship with anabolic effects on bone, muscle mass and increased muscle strength (152, 153). On the other hand, in women the connection is less well established, probably due to ethical concerns such as potential adverse effects of T administration. Additionally, in female athletes there is the obvious problem of exogenous androgens being classified as doping agents and prohibited in sports (8).

In premenopausal women with hypopituitarism, resulting in hypogonadism and/ or adrenal insufficiency, androgen supplementation increased both bone mineral density (BMD) and lean mass compared to placebo (155). Whereas, in postmenopausal women, higher doses of T supplementation (resulting in mean serum T concentration 7.3 nmol/L) increased muscle mass and strength compared to both placebo and lower doses of T (156). Furthermore, in transgender men (biological female to male transgender) receiving T treatment resulting in circulating T levels within the normal male range, muscle mass and muscle strength significantly increase (4, 157).

There are no well-controlled scientific investigations studying androgen supplementation in female elite athletes. However, there is indirect evidence supporting the anabolic effects of androgens, such as the continuous use of AAS among athletes with reports of increased strength and training ability (7). Data concerning women and AAS is limited, except for experiments performed in the former DDR. Documentation revealed that the athletes were subjected to AAS from an early age and that the performance enhancing effects were especially prominent in female athletes (15). In a recently published randomized controlled trial, including healthy, premenopausal, physically active women the effects of T supplementation on body composition and physical performance were evaluated. In the group given exogenous T, serum T levels increased from mean 0.9 to 4.3 nmol/L, and lean mass and aerobic performance increased significantly compared to the placebo group (18) supporting an anabolic effect of exogenous T in women.

3.6.2 Endogenous androgens, body composition and physical performance

In postmenopausal women, high endogenous free T has been associated with a lower prevalence of osteoporotic fractures and positively related to lean mass (158, 159). Also, in non-athletic PCOS women, free T correlated positively to lean mass (145). When starting this PhD project in 2015, there was a lack of scientific studies investigating anabolic effects of endogenous androgens within the normal range in female elite athletes, see table 3. Most previous studies included either a small study group, no control group, analyzed only certain androgens, analyzed T by radioimmunoassay (RIA) and not by mass spectrometry, or did not evaluate physical performance.

Table 3. Studies investigating endogenous androgens, body composition and physical performance in female elite athletes.

Author (citation)	Method	Results
Rickenlund 2003 (138)	Female athletes with menstrual dysfunction (n=25), regularly menstruating female athletes (n=14), sedentary regularly menstruating controls (n=12). Hormonal analyses by RIA, body composition, physical performance.	Female athletes with hyperandrogenism > BMD, beep test, VO_2 max, then female athletes with normal androgen levels. T positively correlated to lean mass and VO_2 max.
Cardinal 2006 (16)	Female elite athletes (n=22). Serum T analyzed by RIA. Physical performance.	Positive correlation between endogenous serum T and CMJ (explosive performance).
Crewther 2010 (17)	Female Olympic weightlifters (n=4). Salivary T analyzed by RIA. Physical performance.	No correlation between pre-work out T and snatch, clean and jerk, and front squat 1RM.
Hagmar 2009 (136)	Female Olympic athletes (n=90). Body composition. Menstrual function. Gynecological ultrasound. Hormonal analyses by RIA.	Most common cause for menstrual dysfunction = PCOS. PCOS athletes had higher androgen levels. Power athletes had significantly higher BMD and lean mass compared to endurance and technical athletes. No data published on correlations between androgens and lean mass.
Bermon 2017 (19)	1332 observations of elite female athletes. T and androstenedione by LC-MS. Best race performance for each athlete recorded.	Female athletes with the higher T tertile had significantly higher performance (1.8-4.5%) in 400 m, 800 m running, hammer throw and compared to female athlete with the lowest T tertile.

1RM=one repetition maximum, BMD=bone mineral density, CMJ=countermovement jump, LC-MS=liquid chromatography mass spectrometry, PCOS=polycystic ovary syndrome, RIA=radioimmunoassay, T=testosterone, VO_2max=maximal oxygen uptake.

Others have compared endogenous T levels between female athletes and controls (160, 161). Tegelman et al (160) found no difference in T levels between female endurance athletes (n= 10) and untrained controls (n=13). In agreement, comparable T levels have been demonstrated between female kayakers (n=21), judokas (n=22) and non-athletic students (n=19) (161). A more recent study, including only nine elite, nine non-elite female athletes and no sedentary controls, found significantly higher salivary T in the elite athletes compared to non-elite athletes (162). Importantly, the immunoassay methods used for determining androgen levels in these studies were not as sensitive and specific as the current gold standard method LC-MS/MS (163) and therefore precursor androgens or androgen metabolites could not be measured.

3.7 ANABOLIC EFFECTS OF THE GH/IGF-I SYSTEM

The GH/IGF-I system has anabolic properties on muscle, bone and on collagen synthesis (tendons, extra cellular matrix) (5, 107, 108, 121, 164, 165). GH per se has lipolytic effects and stimulates gluconeogenesis supplying substrates for growth. These effects may result in improved athletic performance and/or increased training capacity. In GH deficient adults, the anabolic effects of GH replacement therapy are well established, including increased physical performance (164, 166, 167). However, it is questioned if GH/IGF-I supplementation exerts similar performance enhancing effects in healthy subjects (115, 166, 168-170). Even so, professional and recreational athletes use these substances as doping agents (5, 166, 171), despite both GH and IGF-I use being banned from sports by the World Anti-Doping Agency (WADA) (115). For female athletes, the role of endogenous IGF-I for body composition and physical performance is unclear.

3.7.1 Exogenous GH and IGF-I, body composition and physical performance

In adults with GH deficiency, GH supplementation results in increased IGF-I levels and lean mass and reduced fat mass as well as increased maximal oxygen uptake and power output (164, 166, 167). Although exogenous GH given to healthy adults has similar effects on body composition, no increase in aerobic capacity or muscle strength has been demonstrated in this population (164, 168-170).

Two previous randomized controlled trials have examined the effect of GH and IGF therapy in recreational athletes (172, 173). Meinhardt et al (172) found an increase in lean body mass, a decrease in fat mass and a 3.9 % increase in sprint performance after 8 weeks of GH therapy compared to placebo. These effects were even greater in the GH + T supplement group (men only). However, the authors concluded that GH primarily increased extracellular water, while testosterone had a greater influence on body cell mass (the functional compartment of lean body mass) (172). Others found that administration of an IGF-I compound to recreational athletes resulted in no significant changes in body fat or lean body mass, but a significant increase in maximal oxygen consumption compared to placebo (173).

3.7.2 Endogenous IGF-I, body composition and physical performance

To the best of our knowledge no previous studies have examined the relationship between endogenous IGF-I and physical performance in female athletes. However, in untrained women IGFBP-2 has been positively related to peak oxygen uptake (174). In contrast, Eliakim et al (175) found no significant association between IGF-I and aerobic performance.

Three previous investigations have examined IGF-I in relation to body composition in female athletes. In a population of 23 female athletes, Snow et al (176) found significant positive correlations between IGF-I, BMD and lean mass (176). Additionally, Ehrnborg et al (177) measured IGF-I in response to a maximum exercise test and found that IGF-I was positively correlated with weight in 33 female athletes. Lean mass, BMD and fat percent were not measured in this study and potential correlations between exercise test and baseline hormonal data was not reported. In a large study by Healy et al (14), including 92 female athletes, no significant association was found between IGF-I levels and body fat.

Others have compared endogenous IGF-I levels between female athletes and controls demonstrating varying results (176, 178, 179). Limitations of these studies are the low number of participants, and the fact that although IGF-I is known to decrease with age (112, 113), age-adjustment or age-adjusted IGF-I was not reported. The exception is a large study by Healy et al (180), demonstrating higher post-competition endogenous IGF-I levels in elite athletes compared to resting IGF-I levels in non-athletic controls. However, comparison of resting IGF-I levels between athletes and controls was not performed. Furthermore, endogenous IGF-I levels have been shown to vary depending on type of sport (14, 136, 176).

4 RESEARCH AIMS

4.1 GENERAL AIMS

The overall purpose of this thesis was to improve our knowledge of the relationship between endogenous anabolic hormones, body composition, physical performance and the 2D:4D ratio in female elite level athletes. Furthermore, the aim was to evaluate the impact of genetics and hormonal contraceptive use on the urinary steroid profile in athletes and examine the relationship between serum and urinary androgens in women.

4.2 STUDY SPECIFIC AIMS

Study I. To examine the endogenous serum androgen profile in female Olympic athletes compared to controls and between sport categories and in relation to body composition and physical performance.

Study II. To investigate the 2D:4D ratio in female Olympic athletes compared to controls and in relation to endogenous serum and urinary androgens and physical performance.

Study III. To examine endogenous IGF-I, age-adjusted IGF and IGFBP-1 in female Olympic athletes compared to controls, between sport categories and in relation to body composition and physical performance.

Study IV. To evaluate the impact of hormonal contraceptive use and genetic variations on the urinary androgen steroid profile in female elite athletes.

Study V. To investigate the urinary steroid profile in female Olympic athletes compared to controls and in relation to the serum steroid profile and training load.

5 MATERIALS AND METHODS

In the following section the study population is presented in detail, whereas methods are presented focusing on strengths and limitations. A detailed description of each method is presented in previously published papers (75, 181, 182) and manuscripts as part of this thesis and/or in papers cited on the methods applied (39, 40, 112, 183-188).

5.1 STUDY POPULATION AND OVERVIEW OF STUDIES

This thesis includes five studies where studies I, II, III and V are based on the same study cohort consisting of Swedish Female Olympic athletes, n=106 (Swedish Olympic Committee (SOC) study cohort) and untrained controls, n=117. In study IV, 57 of the female athletes participating were part of the SOC study cohort. Written informed consent was given by all participants. In addition, blood and urine samples from 22 female elite athletes that had given consent to participate in research in their anonymous doping control forms were included (Table 4). The studies were approved by the Regional Ethics Committee (Dnr 01-146 and Dnr 2011/1426-32).

Participants in the SOC study cohort were investigated at the Women's Health Research Unit, Karolinska University Hospital or in connection with training camps. Recruitment started in 2011 and continued until 2015 when a representative number of athletes had been included. Female athletes (>18 y), members of a Swedish Olympic team or part of the high-performance program of the SOC were eligible for inclusion. During the recruitment period two Olympic games were held, the summer games in London 2012 and the winter games in Sochi 2014. In total, 106 Swedish female athletes participated in these Olympic games (London n=81, Sochi n=45). For these Olympic team members, 15 did not respond and 5 declined citing a busy schedule and training and competing and living in distant locations as primary reasons. In total, 86 women athletes that participated in the 2012 and 2014 Olympic games were included in the SOC study cohort. In addition, 20 female athletes who were part of the high-performance program of the SOC and/or part of the 2016 Olympic games were included in the study. In the end, a total of 106 female athletes were recruited.

Athletes were divided into sport categories, Power (sport disciplines involving short bursts of intense exertion and high mechanical load), Endurance (sport disciplines involving prolonged periods of submaximal exertion and low mechanical load), Technical (disciplines with a low demand on physical exertion and low mechanical load and focus on technical skills) depending on type of sports (Figure 7) as previously described (182).

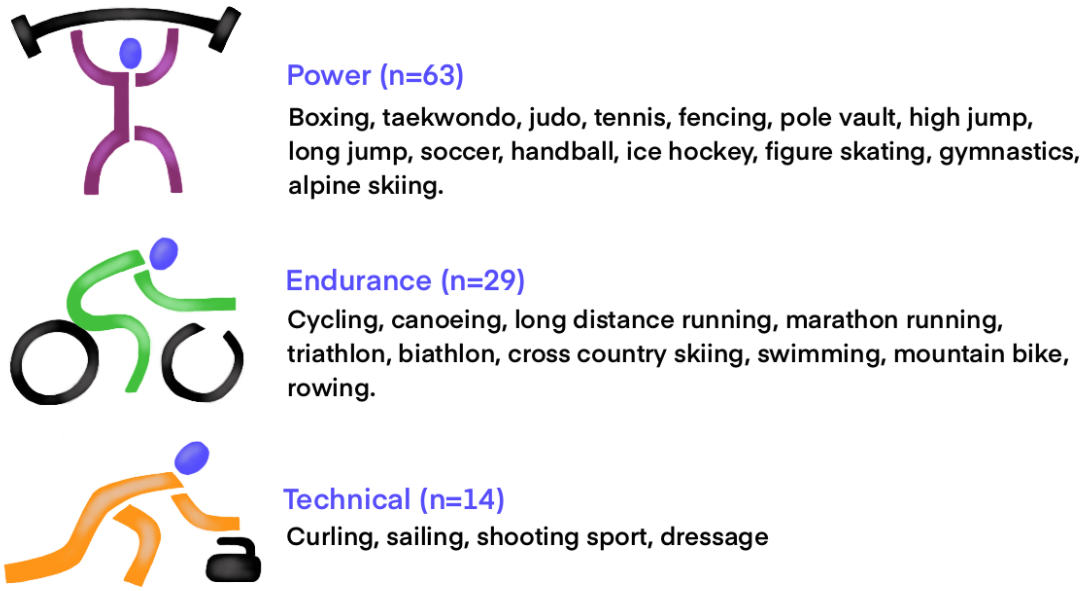

Figure 7: Illustration showing the type of sports in each sport category, power, endurance and technical.

Furthermore, 128 controls were recruited via advertisement at the Karolinska Institutet and online, via a Swedish scientific study recruitment webpage (www.studentkaninen.se). Inclusion criteria were a maximum 2 h training/ week, no prior participation in elite level sports, and age 18-45 years. The controls were initially approached via e-mail, in the order of which they had signed up interest and given written information about the study. Eleven controls were excluded (n=1 prior participation in elite level sports, n=8 training load higher than inclusion criteria, n=2 too high BMI ≥30). In total 117 women controls were included in the study. In table 4, an overview of the studies part of this thesis is presented, including study subjects, study design, methods and the analyses made.

Table 4. Overview of study subjects, study design, methods and analyses made.

Study	Study subjects	Study design	Methods	Analyses
I	106 athletes* 117 controls	Cross-sectional	LC-MS/MS ECLIA DXA SOC physical profile	Serum androgens FSH, LH, SHBG Body composition SJ, CMJ
II	104 athletes* 117 controls	Cross-sectional	Digit 2 and 4 measurements LC-MS/MS DXA SOC physical profile	2D:4D ratio Serum androgens, Urinary steroids# Body composition CMJ, SJ, Bench press, chins, 3000 m running
III	103 athletes* 113 controls	Cross-sectional	RIA DXA SOC physical profile	IGF-I, IGFBP-1 IGFSD calculated Body composition SJ, CMJ, bench press, chins, 3000 m running, squats
IV	57 athletes* 22 athletes	Cross-sectional	GC-MS Genotyping	Urinary steroids UGT2B17, UGT2B7, CYP17A1
V	94 athletes* 86 controls	Cross-sectional	GC-MS/MS Genotyping	Serum androgens, Urinary steroids. UGT2B17

*= The SOC study cohort, #=Glucuronide and sulphate metabolites measured.
CMJ=countermovement jump, CYP=cytochrome P450, DXA=dual-energy X-ray absorptiometry, ECLIA=electrochemiluminescence immunoassay, FSH=follicle-stimulating hormone, GC-MS=gas chromatography-mass spectrometry, GC-MS/MS=gas chromatography-tandem mass spectrometry, IGF-I=insulin- like growth factor-I, IGFSD=age-adjusted IGF-I, IGFBP-1=IGF binding protein-1, LC-MS/MS=liquid chromatography–tandem mass spectrometry, LH=luteinizing hormone, SHBG=sex hormone-binding globulin, SJ=squat jump, SOC=swedish olympic committee, UGT=uridine-diphospho (UDP)-glucuronosyl transferase.

5.1.1 Strengths and Limitations

5.1.1.1 Study design

The studies in this thesis are observational investigations, more specifically we implemented a cross-sectional study design. The main limitation in this type of study is that causality between different outcomes cannot be determined. Instead, cross-sectional studies can identify correlations between variables that can be further investigated. To determine causality, randomized controlled trials are often preferred. However, when conducting research including

elite level athletes, investigating anabolic hormones and the potential impact on physical performance and body composition the researcher is limited to the types of study designs that can be implemented. For example, conducting a randomized controlled trial, supplementing anabolic hormones and investigating the outcome in elite levels athletes would be highly unethical, since anabolic hormones such as androgens and IGF-I are considered to be doping agents and are prohibited from sports by WADA. In addition, our aim was to investigate endogenous anabolic hormones in relation to body composition and physical performance and possible factors that affect the endogenous androgen metabolism, for which a cross-sectional study design is appropriate.

5.1.1.2 Study population

Considering the study population, the sample size was limited by the unique population of interest, i.e. the number of active Swedish Female Olympic athletes available during the recruitment phase. In the end, a large proportion of athletes, 106 Swedish female Olympic athletes part of both the summer and winter games were included, being representative of the study group of interest. For the control group, the aim was to recruit healthy, untrained controls with no previous participation in elite level sport and with similar age and BMI as the athletes. In contrast to the athletes, the controls were recruited from the same geographical area (Stockholm) whereas the athletes were from different areas of Sweden.

When recruiting a study population, one must be aware of possible selection bias. Even though we included a large proportion of Swedish Female Olympic level athletes this group may not be representative of all female Olympic level athletes. However, to our knowledge, this research project is the largest to date including female Olympic level athletes and a control group.

5.1.1.3 Blood and urinary sampling

In order to minimize the possible confounding effects of acute exercise, acute nutritional effects and diurnal rhythm, all blood and urinary samples were collected by a standardized procedure, in a fasted (overnight) and rested (no exercise on test day) state in the morning between 07.00-10.00.

5.1.1.4 Hormonal contraceptive use

A previous study demonstrated that 47% of Swedish Female Olympic athletes used hormonal contraceptives (136). Since there is a limited number of Swedish Female Olympic athletes, excluding the athletes using hormonal contraceptives would have resulted in a significantly

lower number of participants, possibly resulting in loss of statistical power. Furthermore, in study IV, the aim was to evaluate the effects of hormonal contraceptive use on the urinary steroid profile. Therefore, both hormonal contraceptive users and non-users were included. Hormonal contraceptive use was evaluated by questionnaire including yes/no, if yes also type and duration of treatment was determined. In the statistical analyses, hormonal contraceptive use was taken into consideration to minimize possible confounding effects on endocrine parameters.

5.1.1.5 The menstrual cycle

Blood samples were taken randomly during the menstrual cycle. This is acknowledged as a limitation, however, due to logistical reasons, blood testing during a specific cycle phase was not possible. If recruitment had been cycle-dependent, the number of athletes able to participate in the studies with-in a reasonable time period would have been significantly lower. Furthermore, menstrual dysfunction is common among elite athletes (8, 134-136). Therefore, to more accurately reflect female athletes as a population, menstrual dysfunction was not considered an exclusion criterion. In the questionnaire participants were asked if they have regular menstruation and if not, bleeding pattern was established. Menstrual status was defined as: amenorrhea (absence of menstruations during at least the previous 3 months), oligomenorrhea (5-9 periods during the past year, occurring at interval > 6 weeks) or regular menstruation (periods at intervals of 22-34 days). In addition, in regularly menstruating women not using hormonal contraceptives, menstrual cycle phase was confirmed by analyses of serum hormones. Early follicular phase (cycle days 1–7) was defined as E2 <300 pmol/L, progesterone <5.1 nmol/L and low FSH and LH. Ovulatory phase was defined as E2 ≥300 pmol/L, progesterone <5.1 nmol/L and LH higher than FSH. Luteal phase was defined as progesterone >16.9 nmol/L.

5.2 ANALYTICAL METHODS

Hormones were analyzed using mass spectrometry-based methods and/or immunoassays. The serum steroid profile was analyzed at the Endoceutics laboratory, Quebec City, Canada, using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (39, 40). IGF-I, IGFSD and IGFBP-1 were measured by an in-house RIA at the Karolinska Institutet (184, 185). The Urinary steroid profile was established by gas chromatography-mass spectrometry (GC)-MS, GC-MS/MS and LC-MS/MS (186-188) at the WADA accredited anti-

doping laboratory at the Karolinska University Hospital Huddinge, Stockholm. FSH, LH, cortisol, insulin and SHBG were analyzed using standard methods accredited by the Karolinska University Hospital for clinical use. All separate laboratory analyses were conducted on one occasion for the combined group of athletes and controls.

5.2.1 Mass spectrometry-based methods

In this thesis, GC-MS, GC-MS/MS and LC-MS/MS methods were used to establish serum and urinary precursor androgens, active androgens and conjugated androgens. Briefly, these methods rely on initial separation through chromatography, followed by ionization of molecules. The ions then migrate through the mass analyzer allowing separation of ions according to their mass to charge ratio (m/z) using electromagnetic fields. Mass analyzers consists of a series of quadrupoles. In the first quadrupole, ions with a set m/z pass through. These ions (precursor ions) are then fragmented into product ions in a collision cell. In the next quadrupole, the same technique is applied filtrating a second m/z. This tandem mass spectrometry technique allows for better selectivity and sensitivity over single quadrupole instruments. The detector then registers and transforms the signal making it possible in the end to quantify the analyte of interest using different software programs.

Since many compounds have the same molecular mass an initial separation step (differentiating samples depending on the physical characteristics) is needed before applying a mass spectrometric technique. LC separation is dependent on polarity, this is also true for GC but here boiling point has an even greater impact on retention time. Before applying the LC-MS/MS technique, no sample preparation is required besides purifying the samples from proteins. For GC-MS and GC-MS/MS, extraction to organic solvent and derivatization is necessary, see section 5.2.1.2. GC-MS (study IV) and GC-MS/MS (study V) measures the glucuronide conjugated steroids and the unconjugated steroids, whereas LC-MS/MS (study I, II and III) can measure the glucuronide, sulfate and unconjugated fractions of steroids independently.

5.2.1.1 Serum androgen profile

Mass spectrometry-based methods are the reference method for serum steroid analysis although the advanced technical equipment and training required, as well as a relatively high cost meant that immunobased clinical methods were more prevalent in research for many years. In recent years, mass spectrometry analysis of steroids has evolved and become more available, making it possible to accurately measure several steroids in a single analysis (189). In this project, serum precursor androgens, active androgens and androgen metabolites were measured

by a validated LC-MS/MS method, ultra-high-performance LC-MS/MS, at the Endoceutics laboratory, Quebec City, Canada (39, 40, 182). The technique is still relatively expensive compared to immunobased methods. However, when using immunobased methods to measure androgens and T specifically, there is the major problem of antibody cross-reactivity between the structurally similar steroid hormones (precursors, active androgens and metabolites). Since LC-MS/MS does not rely on antibodies, cross-reactivity of steroid compounds is not a confounding factor. Furthermore, in the lower range, as for women, immunobased methods have low accuracy and sensitivity when measuring androgens, such as T. An additional limitation of immunobased measurement of steroid hormones is that the method is restricted to analyze one analyte at a time (4, 189, 190). LC-MS/MS, resulting in highly accurate measurements of androgen levels, is becoming the preferred method in sports endocrinology and many high impact journals only publish scientific papers where androgens have been established by mass spectrometry-based methods (189). Besides LC-MS/MS being more accurate compared to immunoassays, it also offers the possibility to measure the total androgen pool in women including precursor androgens, active androgens and androgen metabolites. Therefore, LC-MS/MS was the chosen method to analyze serum androgens in the studies included in this thesis. A limitation of the mass spectrometry technique is the lack of cross validation between laboratories. However, all serum samples included in the studies of this thesis were analyzed at the same laboratory.

5.2.1.2 Urinary steroid profile

The urinary steroid profile (T-G, EpiT-G, ADT-G, Etio-G, 5αAdiol-G, 5βAdiol-G) was analyzed applying the standard methods used at the WADA accredited doping control laboratory, Karolinska University Hospital, Huddinge, at the time of each study (GC-MS or GC-MS/MS) as previously described (186-188). Therefore, the method for establishing urinary steroids was changed from GC-MS in study IV to GC-MS/MS in study V. To be able to measure both sulphate and glucuronide conjugated steroids in urine in study II, an LC-MS/MS method was used. Furthermore, since the mass spectrometry method cannot distinguish between exogenous and endogenous androgens several biomarker ratios (T/E ratio, androsterone/etiocholanolone (A/Etio) ratio, androsterone/testosterone (A/T) ratio, 5αAdiol/E, 5αAdiol/5βAdiol) have been determined to indirectly measure androgen abuse.

Since the urine samples were stored at -18°C and were subject to several thaw cycles, there is the concern of possible degradation of steroids and bacterial contamination. Taking this into account, during the analytical process following standardized protocols, all samples are checked for bacterial contamination (i.e. presence of metabolites, 5α- and 5β-androstanedione

that can be produced by bacteria but not humans) (68). Before applying the GC-MS and GC-MS/MS technique, sample preparation is required. Briefly this includes the following:

Hydrolysis: Hydrolysis of the glucuronide conjugate by β-glucuronidase (from *E.coli*). Since the hydrolysis time for each steroid differs, an incomplete hydrolysis can result in a decreased A/T and A/Etio ratio (68). Hydrolysis is controlled by evaluating the ratio between d4-A-glucuronide and d5-Etio (both added as surrogate internal standard) and if considered incomplete, the urine sample is prepared once more, controlling for pH, changing the hydrolysis time or increasing the amount of B-glucuronidase.

Extraction: By adding an ether or N-pentane, lipophilic compounds such as unconjugated steroids can be extracted from water, hydrophilic compounds, salts and proteins.

Derivatization: By adding chemical compounds, GC-separation and ionization is improved. An incomplete derivatization may result in a low A/T ratio (68). Derivatization is controlled for by monitoring the androsterone mono-trimethylsilyl derivates (TMS), which should be low compared to the fully reacted bis-TMS. If considered incomplete, additional derivatization reagent could be added and then heated further.

By controlling for hydrolysis, derivatization and bacterial degradation in each sample the quality of the method is maintained. Different methodologies were used to establish the urinary steroid profile in this thesis and even though these methods produce similar results they are not identical. However, since only one method was used to establish urinary steroids for each study, when comparing values within each study the results should not be affected.

5.2.1.3 Specific gravity

The measured steroid concentrations are corrected for the dilution of the urine. Concentrated urine would otherwise overestimate the absolute concentration whereas diluted urine may underestimate the steroid concentration. In doping control samples, specific gravity, the ratio of weight of a volume of urine to the weight of the same volume of pure water at the same temperature, is used to adjust for urine dilution (191, 192). Each sample is corrected to a specific gravity of 1.020 according to: $Ccorrected = Cmeasured * 1.020 – 1/SG – 1$.

5.2.2 Genotyping

Genotyping for UGT2B17, UGT2B7, CYP17A1 was performed at the Department of Laboratory Medicine, KI. DNA was extracted from the whole blood samples using QIAamp® DNA Blood Mini kit (Qiagen). Genotyping was performed using TaqMan genotyping assays and Fast Real-Time polymerase chain reaction (rt-PCR) as previously described (45). Copy

number analysis of UGT2B17 was based on the ΔΔCT method using cycle threshold values compared to a reference gene (193). In study IV, albumin was used as reference gene and in study V, RNaseP. Individuals with a deletion in the UGT2B17 gene, del/del show signal for the reference gene (albumin or RNaseP) but no UGT2B17 amplification. Defining those with one copy vs two copies of the UGT2B17 gene is more complex due to possible signal overlap. Consequently, for some of the study participants UGT2B17 genotype could not be established. Therefore, in study IV, ins/del and ins/ins UGT2B17 individuals were combined as one group.

5.2.1 IGF-I and IGFBP-1

Since GH is released in a pulsatile manner, a single GH measurement does not reflect GH secretion in an accurate way. In contrast, IGF-1 levels are stable throughout the day and is suggested to mediate many of the anabolic effects of GH (5, 115). Therefore, we chose to analyze IGF-I and IGFBP-1, measured by in-house RIAs (184, 185). For detection limit, intra- and inter-assay coefficients of variations (CV) of these methods see table 5. IGF-I circulates bound to IGFBPs, therefore it is necessary to remove the effects of binding proteins in the analyses. Briefly, after separation of IGFBPs, by acid ethanol extraction and cryoprecipitation, IGF-I was determined by RIA. To minimize interference of remaining IGFBPs, des (1-3) IGF-I was used as radio-ligand (184). IGFBP-1 was determined by RIA according to Póvoa et al (185). Circulatory IGF-I levels are age dependent, decreasing with age, taking this into account IGF-I values were also expressed as SD-scores (geometrical mean ± 2 SD) (IGFSD) calculated from the regression of the values of 247 healthy adults according to Hilding et al (112).

It is difficult to compare immunoassay methods between laboratories, especially commercial IGF-I assays, due to the lack of standardization. However, in this thesis, all samples were analyzed for IGF-I using the same method performed at one laboratory. Therefore, difference observed between groups should be valid. In the context of doping, LC-MS/MS methods for analyzing IGF-I have been developed although this is not yet implemented as a standard method and immunoassays are still used.

5.2.2 FSH, LH, SHBG, cortisol, Insulin

Serum levels of FSH, LH, SHBG, Cortisol and Insulin were determined by electrochemiluminescence immunoassay, a standard method accredited by the Karolinska University Hospital. The lower limit of detection and intra- and inter-assay coefficient of variation are presented in table 5.

Table 5. Methods, detection limits, intra- and inter-assay CV for hormonal analyses

Analysis	Manufacturer	Method	Lower DL	Intra-assay CV	Inter-assay CV
FSH	Roche	ECLIA	0.1 E/L	2.6%	3.6%
LH	Roche	ECLIA	0.1 E/L	1.2%	2.0%
SHBG	Roche	ECLIA	0.35 nmol/L	1.3%	2.1%
Cortisol	Roche	ECLIA	1.5 nmol/L	1.7%	2.2%
Insulin	Roche	ECLIA	0.2 mIE/L	1.5%	4.9%
IGF-I	In house	RIA	6 µg/L	4%	11%
IGFBP-1	In house	RIA	3 µg/l	3%	10%

DL=detection limit, ECLIA=electrochemiluminescence immunoassay, FSH=follicle-stimulating hormone, RIA=radioimmunoassay, IGF-I=insulin- like growth factor-I, IGFBP-1=IGFBP-1=IGF binding protein-1, LH=luteinizing hormone, SHBG=sex hormone-binding globulin.

5.3 BODY COMPOSITION

Dual-energy X-ray absorptiometry (DXA) is considered the current reference method for determining body composition (194, 195). It estimates BMD, lean body mass (skeletal muscle, internal organs and intestinal fat) and fat mass for the whole body and also for regional compartments such as the trunk and legs (195). Body composition (fat mass, lean body mass and BMD from the whole body) was measured for 65 athletes (61%) and 100 controls (85%) by DXA, a clinical standard method at the Karolinska University Hospital, Solna using the Lunar Prodigy Advance (GE Healthcare, Madison, WI, USA). The amount of fat in the trunk and legs (limit between defined as the line drawn from the upper margin of the iliac crest to the neck of the femur) was automatically calculated by the software. Upper/lower fat mass ratio was then established. Spinal BMD was established from the whole body DXA. Z-scores were calculated from the mean BMD and their SD values supplied by the manufacturer of the scanner (Z-score <-2SD is defined as low BMD). The reproducibility of the whole body BMD has been calculated to be <0.01 g/cm3 or 0.1xSD (183). Lean mass total % and lean mass legs % were calculated by dividing lean mass for the respective section with total mass and total mass legs, respectively.

5.4 PHYSICAL PERFORMANCE TESTS

In addition, all athletes were offered to perform standardized physical performance tests part of the physical profile of the SOC at the Sports Institute, Bosön, Stockholm. Mainly power athletes participated in the physical performance tests. Furthermore, not all athletes did participate in all physical performance tests, which included squat jump (SJ) (n=59), countermovement jump (CMJ) (n=59), squats (n=50), bench press (n=45), chins (n=49) and 3000 m running (n=20), (Figure 8). For SJ and CMJ maximum height (cm) was recorded using an infrared contact plate, IVAR equipment (IVAR Ltd. Tallinn, Estonia). SJ and CMJ are validated tests for measuring explosive power of the lower limbs (196) and bench press and squats are reliable tests for measuring strength (197).

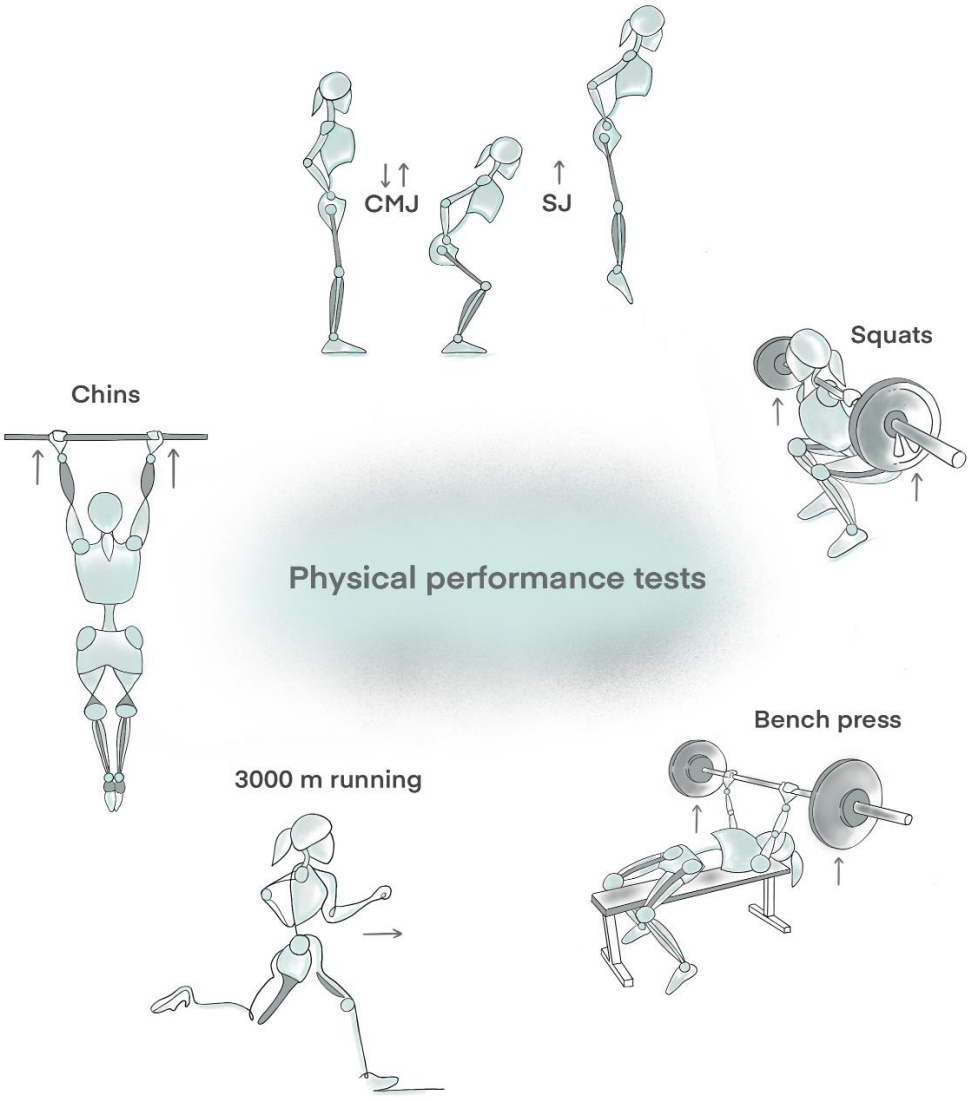

Figure 8: Illustration showing the physical performance tests. CMJ=countermovement jump, SJ=squat jump. ©Emma Eklund.

5.5 2D:4D RATIO

Digit two (2D) and digit 4 (4D) were directly measured on the ventral surface of the hand, from the mid-point of the proximal crease of the proximal phalanx to the distal tip of the distal phalanx, using a Vernier digital caliper 0-150 mm (USA, Cocraft) with a precision of 0.01 (80). Digit measurements were made on both left and right hand and digit ratio calculated by dividing 2D length by 4D length (Figure 5). The digits were independently measured by two raters. For rater A the intraobserver agreement was 0.90 for right hand and 0.90 for left hand. For rater B, the corresponding intraobserver agreement was 0.93 and 0.96, respectively. The inter-rater correlation was 0.87 for the right hand and 0.85 for left hand (198).

5.6 STATISTICAL ANALYSES

Statistical analyses were performed using Statistica version 13 (TIBCO Software inc (2018)) for study I, II, III and V and GraphPad PRISM 6.0® (GraphPad, San Diego, CA, USA) for study IV. The statistical methods used were selected depending on the distribution of the data and presented accordingly. Normally distributed data was analyzed using parametric statistical methods and not normally distributed data analyzed using non-parametric tests or parametric tests following transformation.

Groups were compared employing the unpaired t-test or Mann-Whitney U-test. When appropriate, subgroups were compared using one-way analysis of variance (ANOVA) followed by Fisher, LSD post-hoc analyses, or Kruskal-Wallis ANOVA by ranks. Bonferroni correction was then employed when suitable. Two-way ANOVA was used when comparing groups, evaluating the possible interaction of hormonal contraceptive use and in study I analyses of covariance (ANCOVA) was used controlling for age and HC use. Categorical variables were compared by Chi-square test or Fisher's exact test. Spearman's rank order correlation or Pearson's correlation were employed to assess correlation between variables. In study II, effect size for continuous variables was calculated using Cohen's d and for categorical variables with Phi = √(Chi-2/n).

In study I, a forward stepwise multiple regression analysis was employed to examine to what extent the physical performance tests (SJ and CMJ) could be explained by the serum androgens and lean mass variables. After fitting the regression equation, we examined the residuals by

Cook´s distance. Extreme cases were excluded and the model then reanalyzed. Hormonal contraceptive use was added to the regression models (SJ and CMJ) to evaluate potential interaction with androgens and lean mass variables. P-values <0.05 were considered statistically significant.

5.7 ETHICAL CONSIDERATIONS

Throughout this research project, ethical concerns have been part of the research process and been carefully taken into consideration. All studies were approved by the Regional Ethics Committee (Dnr 01-146 and Dnr 2011/1426-32) at the Karolinska Institute. The participants were volunteers and study participation could be cancelled at any time. When approaching the athletes, we took great care to highlight that participation in the study was voluntary. Controls that presented an interest in the study were given the same information. All participants received written information about the research project and written consent was obtained.

In order to reduce the risk of disclosing sensitive personal information, data from the questionnaire and other variables such as body composition, digit ratio measurements, genotyping, urinary steroid profile or serum hormonal profile was de-identified. Since the study group of interest, i.e. Swedish Female Olympic athletes, is very specific, there is a higher risk of possible identification of study participants and we have constantly been aware of the ethical balancing act between research transparency and the importance of making it impossible to identify individual participants. Therefore, results have been presented and/or published at anonymous group level. Furthermore, the inclusion of study participants was done over several years (2011-2015) minimizing the risk of individual identification.

All sample procedures were explained to the participants and blood samples were taken by medical staff, highly trained in the technique. Body composition was determined by DXA, an examination that does expose the individual to ionizing radiation. However, it is considered a safe procedure and the radiation dose is very low, corresponding to the normal background radiation over one day at sea level (194). All participants had the possibility to get their own results such as serum and urinary steroid hormone profile, genotyping results, body composition and digit ratio measurements. If any data or laboratory value was considered abnormal, a medical follow up was offered at the Department of Obstetrics and Gynecology, Karolinska University Hospital, Solna.

Study participants were genotyped to investigate certain genetic polymorphisms' impact on the urinary steroid profile in women athletes. None of the genetic variations investigated are associated with disease or increased mortality. Furthermore, some results included in this thesis have been published with the aim to optimize the interpretation of the urinary steroid profile. Since the published data can be accessed by medical staff, coaches and athletes this information might be used to avoid being detected in doping controls. However, our research is focused on the anti-doping work and to not publish our results would be unethical, and lead to withholding information we believe can optimize the interpretation of doping tests in women.

6 RESULTS

6.1 GENERAL CHARACTERISTICS OF FEMALE OLYMPIC ATHLETES AND CONTROLS (STUDY I-III)

In table 6, general characteristics are presented for the female Olympic athletes and controls. There was no significant difference in age, BMI or hormonal contraceptive use between groups. However, the athletes were significantly taller (1.71±0.06 vs. 1.68±0.07 cm, p<0.001) and had a higher body weight (64.7±7.5 vs. 61.9±8.4 kg, p<0.01) compared to the controls. As expected, the athletes had significantly higher training hour per week compared to the controls.

Table 6. General characteristics for female Olympic athletes and controls (SOC cohort).

Parameters	Controls	Athletes
n	117	106
Age	26.2±5.5	26.0±5.6
BMI	22.0±2.6	22.0±2.0
HC use, n (%)	46 (39)	41 (39)
MD, n (%)	3 (4)	15 (23)**
Training (hours/week)	0.9±0.9	17.8±5.7***

Values presented as mean ± SD or number and percent.
*p<0.05, **p<0.01, ***p<0.001.
BMI=Body mass index, HC=hormonal contraceptive, MD=menstrual dysfunction. SOC=swedish olympic committee.

Menstrual dysfunction was significantly more common in the athletes (Table 6). In the group of women with regular menstruations, not using hormonal contraceptives, there was no significant difference in cycle phases between groups (Table 7).

Table 7: Cycle phase for regularly menstruating female Olympic athletes and controls not using hormonal contraceptives.

Cycle phase *	Controls (n, %)	Athletes (n,%)
Follicular Phase	24 (39 %)	24 (57 %)
Ovulations Phase	18 (30 %)	8 (19%)
Luteal Phase	19 (31 %)	10 (24 %)
Total	61 (100 %)	42 (100 %)

Number of subjects and percent presented.
* Early follicular phase (cycle days 1–7) was defined as E2 <300 pmol/L, progesterone <5.1 nmol/L and low FSH and LH. Ovulatory phase was defined as E2 ≥300 pmol/L, progesterone <5.1 nmol/L and LH higher than FSH. Luteal phase was defined as progesterone >16.9 nmol/L. For 8 athletes and 7 controls cycle phase could not be determined by hormonal analyses.

6.1.1 Hormonal contraceptives

The majority of women were using contraceptives that inhibit ovulation. Furthermore, the distribution of different types of hormonal contraceptives was comparable between the female Olympic athletes and the controls (Table 8).

Table 8: Hormonal contraceptive type and contraceptive effects among female Olympic athletes and controls.

Type of HC	Controls	Athletes	Substance	Contraceptive effect
HC use	46 (39 %)	41 (39 %)	----	----
COCs	23 (50 %)	27 (66 %)	E + P	Inhibits ovulation
Progestin -pill	3 (7 %)	4 (10 %)	Desogestrel	Inhibits ovulation
Progestin implant	2 (4 %)	4 (10 %)	Etonogestrel	Inhibits ovulation
Progestin IUD	3 (6 %)	1 (2 %)	Levonorgestrel	Endometrial effect
Vaginal ring	10 (22 %)	1 (2%)	E + P	Inhibits ovulation
"yes" HC, ns	5 (11 %)	4 (10 %)	----	----
Inhibits ovulation	38 (83 %)	36 (88 %)	----	----

Number of subjects and percent presented. COCs=combined oral contraceptives, E=estrogen substance, ns=not specified, P=progestin, HC=hormonal contraceptives, IUD=intrauterine device.

The majority of study participants were using COCs. The dose and type of substance for the COCs is presented in table 9.

Table 9: Dose and type of substance in the COCs used by women Olympic athletes and controls.

Dose and substance	Controls (n=23)	Athletes (n=27)
30 µg ethinyl estradiol 0.15 mg levonorgestrel	10 (44 %)	12 (45 %)
20-30 µg ethinyl estradiol 3 mg drosperinone	7 (30 %)	10 (37 %)
30-40 µg ethinyl estradiol 0.05-0.125 mg levonorgestrel*	0	1 (4 %)
35 µg ethinyl estradiol 0.25 mg norgestimat	2 (10 %)	2 (7%)
35 µg ethinyl estradiol 2.0 mg cyproterone	1 (4 %)	0
30 µg ethinyl estradiol 0.5-1.0 mg noretisterone	1 (4 %)	2 (7%)
1-3 mg estradiol 2-3 mg diegonest	1 (4%)	0
1.5 mg estradiol 2.5 mg nomegestrol	1 (4%)	0

Number and percent presented. COCs=combined oral contraceptives, n=number. *=triphasic.

6.2 SPORT CATEGORIES

The athletes were divided into sport categories, power (n=63), endurance (n=29) and technical (n=14), depending on type of sport (Figure 7), as previously described by Hagmar et al (136).

6.2.1 General characteristics, hormonal contraceptive use and menstrual dysfunction

Age, BMI and training hours per week differed between the athlete groups. No significant differences were found regarding the frequency of hormonal contraceptive use or menstrual dysfunction between sport categories (Table 10).

Table 10. General characteristics for female Olympic athletes of different sport categories (Power, Endurance, Technical).

Parameters	Power	Endurance	Technical
n	63	29	14
Age	25.2±4.3	24.8±4.3	32.1±8.9 b***c***
BMI	22.1±1.9	21.3±1.4	23.3±3.0 c**
HC use, n (%)	23 (37)	13 (45)	5 (36)
MD, n (%)	7 (18)	6 (38)	2 (22)
Training hours (h/w)	16.6±5.8	20.7±4.5	17.1±5.6 a**c*

Values presented as mean ± SD or number and percent.
*p<0.05, **p<0.01, ***p<0.001.
a, power vs endurance; b, power vs technical; c, endurance vs technical
BMI=body mass index, HC=hormonal contraceptives, MD=menstrual dysfunction.

6.3 ANABOLIC HORMONES

6.3.1 Anabolic hormones in athletes and controls

Serum levels of the precursor androgens DHEA and 5-DIOL and the androgen metabolite Etio-G were significantly higher among the athletes compared to controls, whereas estrone was significantly lower (Table 11). Serum T levels were within normal range for all study participants and no significant difference was observed between groups. Similar results were found for DHT (102 (85-150) vs. 102 (77-137) pg/mL) for controls and athletes, respectively. In addition, the athletes demonstrated significantly higher serum IGF-I, IGFSD and IGFBP-1 and significantly lower insulin levels compared to controls (Table 11).

Table 11. Endocrine profile for female Olympic athletes and female controls (SOC cohort).

Endocrine profile	Controls	Athletes
n	117	106
E1, pmol/L	165.0 (95.4-273.3)	130.9 (85.1-211.9)*
E2, pmol/L	174.4 (79.7-436.9)	130.7 (52.5-297.7)
T, nmol/L	0.992±0.35	0.995±0.40
SHBG, nmol/L	77.0 (54.0-139.0)	83.5 (58.0-125.0)
DHEA, nmol/L	19.1 (15.3-27.8)	25.3 (15.6-36.8) **
5-DIOL, pg/mL	626±237	713±301*
Etio-G, ng/mL	23.7 (17.5-33.3)	30.0 (19.7-41.9) *
n	113	103
IGF-I, µg/L^	249.7±73.3	277.5±85.5*
IGFSD^	-0.18±1.08	0.15±1.03*
IGFBP-1, µg/L ^	49 (29-72)	62 (42-79)**
Insulin, mIE/L^	7.6 (5.6-9.8)	6.0 (4.5-8.1)***

Values presented as mean ± SD or median or interquartile range (25^{th}–75^{th} percentile).
*p<0.05, **p<0.01, ***p<0.001.
5-DIOL=androstenediol, DHEA=dehydroepiandrosterone, E1=estrone, E2=estradiol Etio-G=etiocholanolone glucuronide, IGF-I=insulin-like growth factor-I, IGFBP-1=insulin-like growth factor binding protein-1, IGFSD=insulin-like growth factor-I age-dependent reference range, SHBG=sex hormone-binding globulin, SOC=swedish olympic committee, T=testosterone.
^ = one athlete with diabetes type 1 excluded from analyses
To convert: T nmol/L to ng/dL divide by 0.0347, DHEA nmol/L to ng/mL divide by 3.467, E1 pmol/L to pg/mL divide by 3.699, E2 pmol/L to pg/mL divide by 3.671.

Hormonal contraceptive use showed no statistically significant interaction when comparing serum androgens (DHEA, 5-DIOL, T, DHT, Etio-G) and IGF-I, IGFSD and IGFBP-1 between athletes and controls. However, as expected, in the total population of athletes and controls, sex hormone levels were significantly lower in hormonal contraceptive users, whereas SHBG and IGFBP-1 were significantly higher (Table 12). 5-DIOL, IGF-I and IGFSD were comparable between hormonal contraceptive users and non-users.

Table 12. Serum hormone levels depending on hormonal contraceptive use in the total population of female Olympic athletes and controls (SOC cohort).

Parameters	Non-HC users	HC users
n	136	87
FSH, E/L	4.5 (3.2-6.1)	3.7 (0.8-6.0)**
LH, E/L	6.5 (4.6-9.7)	2.8 (0.6-6.5)***
E2, pmol/L	315.7 (130.3-533.4)	176.6 (48.5-457.8)***
T, nmol/L	1.05±0.36	0.91±0.38**
SHBG, nmol/L	70.0 (49.0-90.0)	136.0 (79.0-203.0)***
A4, nmol/L	5.0±1.8	3.5±1.4***
DHEA, nmol/L	23.3 (16.3-33.7)	21.2 (13.9-25.7)**
IGFBP-1, µg/L	50.3±29.4	71.9±35.4***

Values presented as mean ± SD or median and interquartile range (25^{th}–75^{th} percentile).
*p<0.05, **p<0.01, ***p<0.001.
A4=androstenedione, DHEA=dehydroepiandrosterone, E2=estradiol, FSH=follicular-stimulating hormone, HC=hormonal contraceptives, IGFBP-1=insulin-like growth factor binding protein-1, LH=luteinizing hormone, SHBG=sex hormone-binding globulin, SOC=swedish olympic committee T=testosterone.
To convert: T nmol/L to ng/dL divide by 0.0347, DHEA nmol/L to ng/mL divide by 3.467, E1 pmol/L to pg/mL divide by 3.699, E2 pmol/L to pg/mL divide by 3.671, A4 nmol/L to ng/dL divide by 0.0349.

6.3.2 Anabolic hormones depending on sport category

Serum androgens and estrogens were comparable between groups. The power athletes had significantly higher IGF-I compared to technical athletes and significantly higher IGFSD compared to both endurance and technical athletes (Table 13). No statistically significant interaction was found for hormonal contraceptive use or age when comparing serum androgens, estrogens, IGF, IGFSD and IGFBP-1 between sport categories.

Table 13. IGF-I and IGFSD female Olympic athletes of different sport categories (Power, Endurance, Technical).

Endocrine profile	Power	Endurance	Technical
n	62	29	12
IGF-I µg/L	293.6±78.4	264.4±94.3	221.5±76.2 b**
IGFSD	0.37±0.88	-0.11±1.18	-0.44±1.06 a*b*

Values presented as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001.
a, power vs endurance; b, power vs technical; c, endurance vs technical
IGF-I=insulin-like growth factor-I, IGFSD=age adjusted IGF-I.

6.4 2D:4D RATIO (STUDY II)

The 2D:4D ratio right hand was significantly lower in the female Olympic athletes compared to female controls (Table 14). 2D:4D ratio left hand was comparable between groups.

Table 14. 2D:4D ratio for female Olympic athletes and female controls.

2D:4D ratio	Controls	Athletes
n	117	104
Right 2D:4D ratio	0.98±0.04	0.97±0.03*
Left 2D:4D ratio	0.97±0.03	0.97±0.03

Values presented as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001.
2D:4D ratio=second to fourth digit ratio.

6.4.1 Correlations between the 2D:4D ratio and the urinary steroid profile

In the athletes, there were significant negative correlations between the 2D:4D ratio right hand and the urinary steroid metabolites, T-G (r_s= -0.25, p <0.05), 5αAdiol-17G (r=-0.23, p<0.05) and 5βAdiol-17G (r_s= -0.21, p<0.05), i.e. the lower digit ratio the higher androgen metabolites. There were no significant correlations between the 2D:4D ratio and serum androgen levels in the athletes or controls.

6.5 BODY COMPOSITION

6.5.1 Body composition in athletes and controls

In table 15, body composition data is presented for all athletes and controls that participated in the DXA examination. As expected, the athletes had a more anabolic body composition with higher BMD and lean mass and lower body fat percent.

Table 15. Body composition, including bone mineral density, body fat percent and lean body mass for female Olympic athletes and controls.

Body Composition	**Controls**	**Athletes**
n	100	65
Total BMD g/cm^2	1.15±0.07	1.25±0.08***
Spinal BMD g/cm^2	1.01±0.10	1.11±0.11***
Z-score	0.35±0.86	1.60±1.02***
Body fat percent (%)	31.7±6.6	18.4±5.9***
Lean mass total (kg)	40.4±4.1	49.9±5.9***
Lean mass legs (kg)	13.6±1.6	17.3±2.2***

Values presented as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001.
BMD=bone mineral density, kg=kilograms.

6.5.2 Body composition depending on sport category

The power athletes had the highest BMD and Z-score compared to both technical and endurance athletes. The technical athletes had the highest body fat percent whereas endurance athletes had the lowest body fat percent. Power and endurance athletes had significantly higher lean body mass compared to technical athletes. The endurance athletes had the highest percentage of total lean body mass compared to the other sport categories (Table 16).

Table 16. Body composition, including bone mineral density, body fat percent and lean body mass for female Olympic athletes of different sport categories (Power, Endurance, Technical).

Body composition	Power	Endurance	Technical
n	42	18	5
Total BMD g/cm^2	1.28±0.07	1.19±0.07	1.17±0.05 a***b**
Spinal BMD g/cm^2	1.14±0.10	1.06±0.12	1.02±0.07 a*
Z-score	2.04±0.85	0.82±0.85	0.76±0.64 a***b**
Body fat percent (%)	19.3±5.3	14.4±4.7	25.3±6.0 a**c**
Lean mass total (kg)	49.4±5.9	53.0±4.3	43.2±2.9 b*c**
Lean mass legs (kg)	17.4±2.4	17.8±1.8	14.4±1.4 b*c**

Values presented as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001.
a, power vs endurance; b, power vs technical; c, endurance vs technical
BMD=bone mineral density.

6.6 CORRELATIONS BETWEEN SERUM HORMONES AND BODY COMPOSITION

6.6.1 Serum Androgens

In the athletes, serum androgens were significantly positively correlated with total BMD (T (r_s=0.31, p<0.05), DHEA (r_s=0.28, p<0.05), A (r_s=0.27, p<0.05)) and Z-score (T (r_s=0.35, p<0.01), DHEA (r_s=0.29, p<0.05), A (r_s=0.30, p<0.05)). Furthermore, the precursor androgens 5-DIOL and DHEA correlated positively to lean mass (Table 17). Similar findings were observed in the subgroup of athletes not using hormonal contraceptives (Table 17). Etio-G was significantly positively correlated to lean mass total (%) (r_s=0.31, p<0.05) and lean mass legs (%) (rs=0.32, p<0.01).

Table 17: Correlations between endogenous androgens and lean mass for female Olympic athletes not using HC and for the total group of athletes.

	Athletes not using HC (n=41)		Athletes total (n=65)	
Androgens	DHEA	5-DIOL	DHEA	5-DIOL
Lean mass total	**r_s = 0.44**	**r_s = 0.34**	**r_s = 0.27**	r_s = 0.16
	p = 0.004	**p = 0.031**	**p = 0.03**	p = 0.196
Lean mass legs	**r_s = 0.49**	**r_s = 0.39**	**r_s = 0.33**	**r_s = 0.25**
	p = 0.001	**p = 0.012**	**p = 0.007**	**p = 0.041**

Spearman correlation coefficient (r_s) and p-values presented. Significant data marked in bold text. 5-DIOL=androstenediol, DHEA=dehydroepiandrosterone, HC=hormonal contraceptives.

6.6.2 IGF-I

In the total population of athletes and controls, IGFSD was significantly positively correlated with height (r_s=0.14, p <0.05), total BMD (r_s=0.23, p <0.01), Z-score (r_s=0.23, p <0.01) and lean mass legs (kg) (r_s=0.16, p <0.05). IGFBP-1 was positively correlated with spine BMD (r_s=0.20, p <0.05), lean mass total (r_s=0.20, p <0.05) and lean mass legs (r_s=0.20, p <0.05) and negatively correlated with body fat percent (r_s= -0.16, p <0.05). Insulin was negatively correlated to total BMD (r_s= -0.20, p <0.05), Z-score (r_s= -0.27, p <0.001) and lean mass total (kg) (r_s=-0.20, p <0.01) and positively correlated to fat percent (r_s=0.28, p <0.001).

In the athletes, IGF-I and IGFSD were significantly positive correlated with height (r_s = 0.21, p <0.05, r_s=0.21, p<0.05, respectively). Furthermore, IGFBP-1 was significantly correlated with total BMD (r_s=0.28, p= <0.05) and spine BMD (r_s=0.28, p= <0.05).

6.7 CORRELATIONS BETWEEN SERUM HORMONES AND PHYSICAL PERFORMANCE

6.7.1 Serum androgens

In the athletes, DHEA and 5-DIOL were significantly positively correlated with SJ and CMJ (Figure 9). In addition, serum levels of Etio-G correlated positively with SJ (r_s =0.26, p <0.05) and DHT with both SJ and CMJ (r_s=0.27, p <0.05 and r_s=0.39, p<0.01, respectively). In the subgroup of athletes not using hormonal contraceptives, most of these correlations remained significant (Table 18). Lean mass was positively correlated with SJ and CMJ. To evaluate the association between lean mass, the serum androgens and SJ and CMJ a multiple regression analyses was applied. The regression model demonstrated that the strongest factor predicting SJ was DHEA (β = 0.59, p <0.001), then lean mass legs (β = 0.42, p <0.001), in total accounting for 66 % of the variance in SJ. For CMJ, lean mass legs (β =0.65, p <0.001) and lean mass total (%) (β = 0.40, p <0.01) explained 52% of the variance. Hormonal contraceptives did not show any statistically significant interaction with lean mass or DHEA when added to the regression model.

Table 18: Correlations between endogenous hormones, CMJ and SJ in female Olympic athletes not using HC and for the total group of athletes.

	Athletes not using HC		Athletes total	
Performance test	SJ	CMJ	SJ	CMJ
Number	37	38	59	59
DHEA	**r_s = 0.37** **p = 0.026**	**r_s = 0.41** **p = 0.011**	**r_s = 0.39** **p = 0.002**	**r_s = 0.36** **p = 0.005**
5-DIOL	r_s = 0.28 p = 0.093	**r_s = 0.39** **p = 0.015**	**r_s = 0.34** **p = 0.009**	**r_s = 0.37** **p = 0.004**
DHT	r_s = 0.32 p = 0.055	**r_s = 0.47** **p = 0.003**	**r_s = 0.27** **p = 0.037**	**r_s = 0.39** **p = 0.002**

Spearman correlation coefficient (r_s) and p-values presented. Significant data marked in bold text. 5-DIOL=androstenediol, CMJ=countermovement jump, DHEA=dehydroepiandrosterone, DHT=dihydrotestosterone, HC=hormonal contraceptives, SJ=squat jump.

6.7.2 IGF-I

In the athletes, serum levels of IGF-I were significantly positively correlated with SJ, whereas IGFBP-1 correlated positively with squats (Figure 9). In the subgroup of athletes not using hormonal contraceptives the correlation between IGFBP-1 and squats remained.

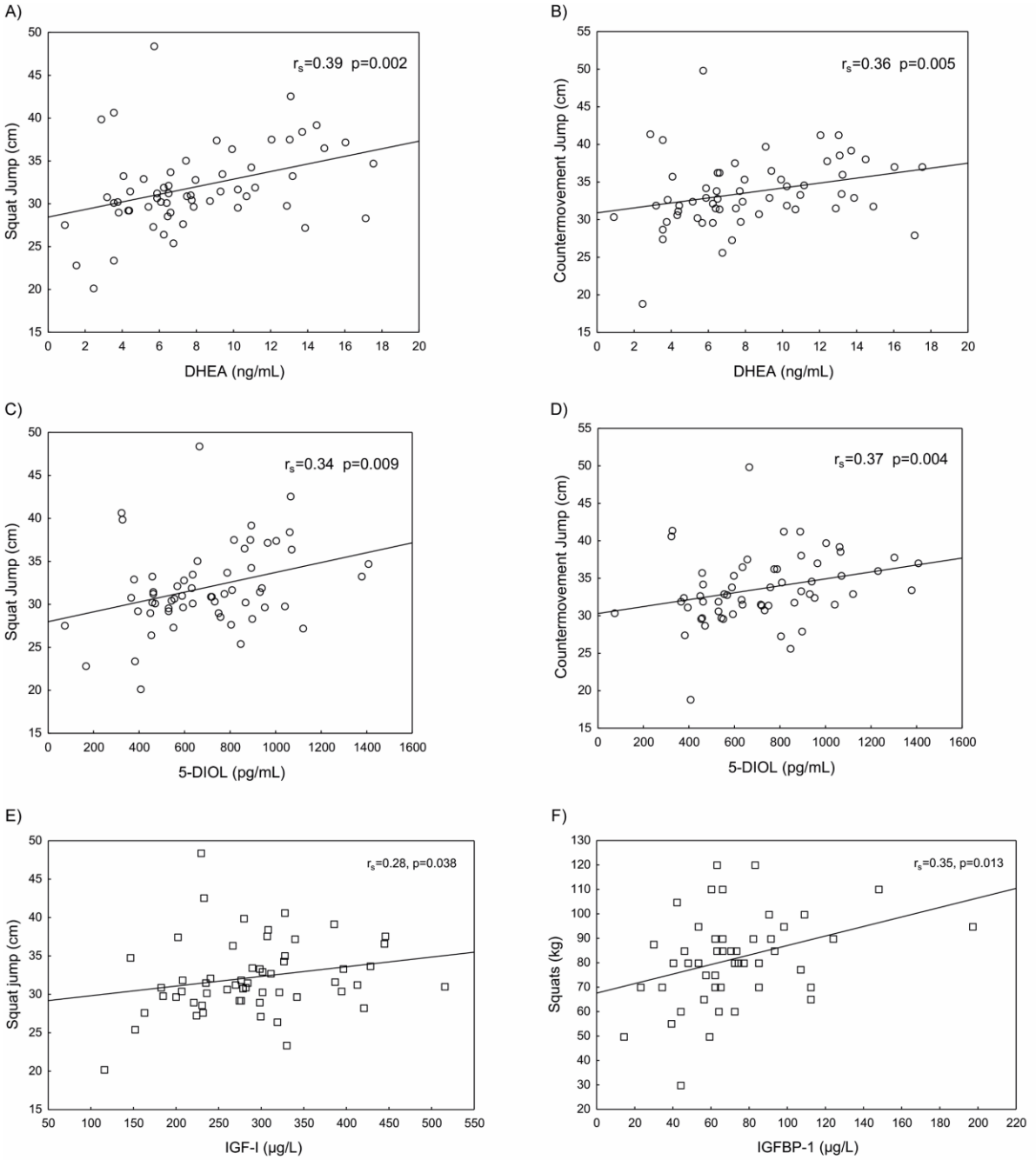

Figure 9: Correlations of serum precursor androgens, IGF-I and IGFBP-1 with physical performance tests in female Olympic athletes. A) Relationship between DHEA and squat jump, B) between DHEA and countermovement jump, C) between 5-DIOL and squat jump, D) between 5-DIOL and countermovement jump, E) between IGF-I and squat jump and F) between IGFBP-1 and squats. 5-DIOL=androstenediol, DHEA=dehydroepiandrosterone, IGF-I=insulin-like growth factor-I, IGFBP-1=insulin-like growth factor binding protein-1. Reproduced from publication Serum androgen profile and physical performance in women Olympic athletes, E Eklund, B Berglund, F Labrie, Carlström K, Ekström L, Hirschberg AH, 2017;51(17):1301-8, copyright notice year 2017. With permission from BMJ Publishing Group Ltd.

6.7.3 2D:4D ratio and physical performance

The 2D:4D ratio right hand was significantly negatively correlated with bench press (r= -0.36, p=0.015) and chins (r_s= -0.28, p=0.050) and significantly positively correlated with 3000 m running performance time (r=0.51, p=0.022).

6.8 THE URINARY STEROID PROFILE IN FEMALE ATHLETES AND CONTROLS (STUDY IV AND V)

6.8.1 General characteristics of female athletes (study IV)

In total 79 female athletes participated in the study with a mean age of 26, ranging from 18-45 years. Hormonal contraceptive use was evaluated by questionnaire (n=57) or from the anonymous doping control form (n=22). In total, 25 (32 %) of the athletes used hormonal contraceptives, the majority taking COCs (n=23). Progestin only contraceptives (progestin intrauterine device or desogestrel) were used by two women.

6.9 THE IMPACT OF GENETICS AND HORMONAL CONTRACEPTIVES ON THE URINARY STEROID PROFILE

6.9.1 Genetics

The allele frequencies for the investigated polymorphisms (UGT2B17, UGT2B7 and CYP 17A1) were similar for study IV and study V and in line with previously published data in Caucasians.

In study IV, the UGT2B17 genotype had a significant impact on the urinary T levels as well as the T/E ratio in female athletes. The UGT2B17 del/del individuals excreted significantly lower levels of urinary T and had a significantly lower T/E ratio compared to the combined ins/del and ins/ins group. Furthermore, the 5α-diol/5β-diol ratio was significantly higher in the UGT2B17 del/del athletes (Table 19). The A/Etio ratio and A/EpiT ratio did not differ between groups. These results were verified in study V, both in female Olympic athletes and female controls. In addition, there were significant differences in U-T and T/E between all UGT2B17 groups (Table 19).

Table 19: Comparison of the urinary steroid profile depending on UGT2B17 genotype in female athletes and controls.

Study IV, Female athletes **(**Del/Del n=6, Ins/Del n=40, Ins/Ins n=22)			
UGT2B17	**Del/Del**	**Ins/Del + Ins/Ins**	**p-value**
U-T ng/mL	0.66 (0.46-1.5)	5.4 (0.74-19.4)	***
U-EpiT ng/mL	4.9 (1.8-21.5)	6.6 (1.2-25)	ns
T/E	0.15 (0.09-0.3)	0.8 (0.8-3.4)	***
5α-diol/5β-diol	0.99 (0.45-1.8)	0.32 (0.06-1.0)	**
Study V, Female Olympic athletes (Del/Del n=9, Ins/Del n=45, Ins/Ins n=39)			
UGT2B17	**Del/Del**	**Ins/Del**	**Ins/Ins**
U-T ng/mL	0.43 (0.29-0.52)	4.24 (2.25-5.82)	6.03 (4.24-10.74) a***b***c***
U-EpiT ng/mL	5.83 (3.15-12.56)	6.55 (3.17-11.41)	6.03 (3.96-10.73)
T/E ratio	0.06 (0.05-0.09)	0.64 (0.40-1.02)	1.18 (0.70-1.71) a***,b***,c***
5α-diol/5β-diol	0.76 (0.67-1.32)	0.27 (0.17-0.44)	0.24 (0.12-0.41) a***,b***
Study V, Female controls (Del/Del n=8, Ins/Del n=45, Ins/Ins n=32)			
UGT2B17	**Del/Del**	**Ins/Del**	**Ins/Ins**
U-T ng/mL	0.69 (0.48-0.87)	6.41 (4.06-10.20)	14.41 (5.64-19.78) a**b***c**
U-EpiT ng/mL	13.83 (8.86-22.07)	10.81 (5.52-14.93)	13.06 (6.50-23.68)
T/E ratio	0.05 (0.03-0.08)	0.66 (0.43-1.01)	0.90 (0.69-1.23) a***,b***,c*
5α-diol/5β-diol	0.70 (0.51-1.05)	0.35 (0.23-0.51)	0.27 (0.14-0.58) a*,b**

Study IV, median and range. Study V, median and interquartile range (25^{th}-75^{th} percentile). Urinary steroids adjusted for specific gravity.
*p<0.05, **p<0.01, ***p<0.001.
a, Del/Del vs Ins/Del: b, Del/Del vs Ins/Ins and c, Ins/Del vs Ins/Ins.
5α-diol=5α-Androstane-3α,17β-diol 5β-diol=5β-Androstane-3α,17β-diol ns=non-significant, T/E ratio=testosterone/epitestosterone ratio, U-EpiT=urinary epitestosterone, U-T=urinary testosterone.

In study IV, the UGT2B7 polymorphism was not associated with any significant difference in urinary steroid levels. For the CYP17A1 polymorphism, in the subgroup of hormonal contraceptive users, individuals with the CC+CT genotype had significantly higher urinary EpiT (5.6 (1.6–12.9) vs. 2.2 (0.91–5.9) ng/mL, p<0.01, respectively) and T levels (6.2(2.3–14.4) vs. 2.4 (0.74–9.4) ng/mL, p<0.05, respectively) compared to the TT genotype group. In the athletes not using hormonal contraceptives, there were no significant differences in urinary steroid levels depending on CYP17A1 genotype.

6.9.2 Hormonal contraceptives

In study IV, female athletes using hormonal contraceptives had significantly lower urinary EpiT levels compared to non-users (4.9 (range: 0.90–12.9) ng/mL vs. 8.2 (range:1.5–25.0) ng/mL, p <0.0005, respectively)). When removing individuals with a UGT2B17 deletion (n=6) the T/E ratio was significantly higher in hormonal contraceptive users compared to non-users (0.9 (range: 0.39–3.4) vs. 0.7 (range: 0.08–2.8) ng/mL, p<0.05, respectively)) (Figure 10). The A/EpiT was significantly higher in hormonal contraceptive users. These findings were verified in the athletes and controls included in study V (data not shown). In addition, in both athletes and controls after excluding del/del individuals, U-T levels were significantly lower in hormonal contraceptive users (3.3 (1.7-5.4) and 5.5 (4.1-10.2) ng/mL) compared to non- users (5.1 (2.7-9.0) and 7.0 (4.3-16.3) ng/mL, p <0.05 and p <0.001, respectively).

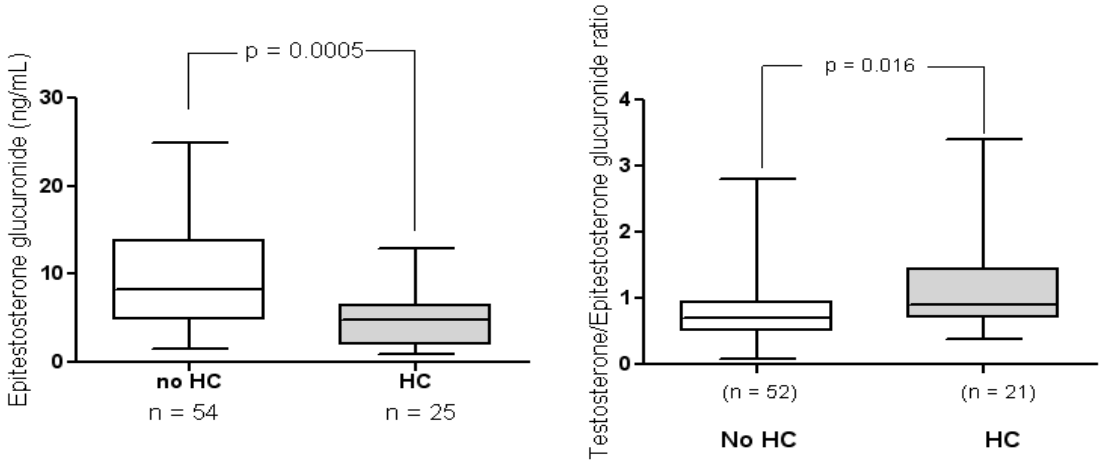

Figure 10: Urinary epitestosterone levels in female athletes not using hormonal contraceptives (no HC) compared to those taking hormonal contraceptives (HC) and Testosterone/Epitestosterone glucuronide ratio in female athletes depending on HC use after removing the UGT2B17 del/del individuals (n=6). Reprinted from Frontiers in Endocrinology, vol 5, article 50, p 1-6; copyright 2014; Open access.CC-BY license (181).

6.10 COMPARISON OF THE URINARY STEROID PROFILE BETWEEN FEMALE OLYMPIC ATHLETES AND CONTROLS (STUDY V)

The female Olympic athletes demonstrated significantly lower urinary androgen metabolite levels compared to the controls, although serum androgen levels were comparable. The A/Etio ratio was significantly lower in the athletes than the controls, whereas all other ratios part of the ABP were comparable between groups as well as serum T, SHBG and FAI (Table 20). In the subgroup of athletes and controls not using hormonal contraceptives similar results were

found (data not shown), except for cortisol being higher in the subgroup of athletes not using hormonal contraceptives compared to controls not using hormonal contraceptives (474.1 ± 131.2 vs. 376.4 ± 108.0, p=0.004).

Table 20: Serum hormones and urinary androgen metabolites in female Olympic athletes and controls.

Serum hormones	**Controls**	**Athletes**
n	86	94
T (nmol/L)	1.0 ± 0.37	0.99 ± 0.40
SHBG (nmol/L)	80.5 (62.0-129.0)	82.0 (57.0 -117.0)
FAI	1.1 (0.6-1.8)	1.1 (0.6-1.9)
Cortisol (nmol/L) ^	516.0 ± 263.6	579.0 ± 216.3
U-androgen metabolites		
U- Testosterone (ng/mL)	6.90 (4.27-14.30)	4.59 (2.25-8.00)***
U- Epitestosterone (ng/mL)	10.99 (6.34-19.64)	6.09 (3.60-11.41)***
U- Androsterone (ng/mL)	3386 (2390 -5627)	2178 (1278 -3554)***
U- Etiocholanolone (ng/mL)	3647 (2504 -4838)	2762 (1769 -4139)**
U- 5αAdiol (ng/mL)	33.4 (21.3-52.7)	19.6 (12.4-30.0)***
U- 5βAdiol (ng/mL)	86.8 (53.0-197.1)	84.5 (39.1-132.8)*
T/E ratio ^	0.7 (0.5-1.1)	0.7 (0.4-1.3)
A/Etio ratio	1.0 (0.8 -1.3)	0.8 (0.6-1.1)*
A/T ratio	458 (294-767)	428 (329 -696)
5αAdiol/E	3.2 (1.9-5.0)	3.6 (2.4-4.8)
5αAdiol/5βAdiol	0.3 (0.2-0.6)	0.3 (0.2-0.5)

Values presented as mean ± SD or median and interquartile range (25^{th}-75^{th} percentile).
*p < 0.05, **p < 0.01, ***p < 0.001.
5αAdiol=U-5α-Androstane-3α,17β-diol, 5βAdiol=U-5β-Androstane-3α,17β-diol, A=androsterone, E=epitestosterone, Etio=etiocholanolone, FAI=free androgen index, SHBG= sex hormone-binding globulin, T=testosterone.
^= Two-way ANOVA indicated that HC use interacted with the comparison between groups.

6.10.1 Correlations between training load and the urinary steroid profile

As expected, the athletes had significantly higher training hour per week compared to controls (18.2 ±5.8 h vs. 0.7 ±0.8, p <0.001, respectively). In the athletes, urinary steroid metabolites: androsterone (r_s=-0.28, p=0.007), epitestosterone (r_s=-0.22, p=0.034), 5αAdiol (r_s=-0.31, p=0.002) and testosterone (r_s=-0.24, p=0.026), were negatively correlated with training frequency (hours per week).

7 DISCUSSION

In this thesis, anabolic hormones were compared between female elite athletes and controls, between sport categories and in relation to body composition and physical performance. The 2D:4D ratio was also investigated in athletes and controls and in relation to serum and urinary androgen levels and physical performance. Furthermore, the impact of hormonal contraceptives and genetic variation on the urinary steroid profile was examined in female elite athletes and the urinary steroid profile compared between athletes and controls and in relation to training hours per week. **In study I**, precursor androgens were significantly higher in the athletes compared to controls and were related to a more anabolic body composition and increased physical performance in the athletes. **In study II**, the 2D:4D ratio was found to be lower in the athletes than controls and related to the urinary steroid profile and physical performance, but not to serum androgen levels. **In study III**, the athletes demonstrated higher IGF-I, IGFSD and IGFBP-1 compared to controls and power athletes had the highest IGFSD compared to endurance and technical athletes. IGF-I was positively associated with lean mass and certain physical performance tests. **In study IV**, hormonal contraceptive use was associated with significantly lower urinary EpiT-G levels and when removing del/del individuals the T/E ratio was higher in athletes using hormonal contraceptives. The UGT2B17 deletion polymorphism and CYP17A1 promoter polymorphism also influenced the urinary steroid profile in female athletes. **In study V**, athletes were found to excrete approximately 30% lower urinary androgen metabolites compared to controls, although serum levels were comparable. In the athletes, the urinary steroid metabolites were negatively correlated with training hours per week.

7.1 ANABOLIC HORMONES IN FEMALE ELITE ATHLETES AND CONTROLS

There is limited previous knowledge on endogenous androgens and IGF-I in female elite athletes compared to untrained controls. In contrast to most previous studies, we measured serum androgens using LC-MS/MS allowing for precise determination of androgen levels in women and the possibility to measure precursor androgens, active androgens and androgen metabolites. In study I, serum T levels were within the normal range for all participants and did not differ significantly between groups, in agreement with two previous publications (160, 161). However, Rickenlund et al (138) found higher T levels in a subgroup of endurance

athletes with menstrual disturbance than in sedentary controls. In our population, the subgroup of athletes with menstrual dysfunction had significantly higher serum Etio-G compared to athletes with regular menstruation and controls.

Furthermore, we found significantly higher serum levels of precursor androgens (DHEA, 5-DIOL) and the androgen metabolite (Etio-G) in the athletes compared to controls. In women, most active androgens are synthesized by transformation of precursor androgens, such as DHEA, in the target tissue (20, 23, 25, 26). It is possible that the higher levels of precursor androgens found in the athletes result in more substrate for intra-cellular transformation to active androgens able to exert anabolic effects. To further examine the anabolic hormonal profile in female elite athletes, IGF-I, age-adjusted IGF and IGFBP-1 were investigated in study III. The higher IGF-I and IGFBP-I levels in our population of female athletes compared to the controls is in agreement with Roli et al (178) reporting higher calculated IGF-I reference ranges in 58 female volleyball players compared to that of a female reference population. In contrast, others have reported no significant difference in IGF-I levels between female athletes and controls, although the study populations were small (176, 179).

The underlying mechanism for our observed findings of increased endogenous precursor androgens and IGF-I in the athletes in not known and owing to the cross-sectional study design, causality cannot be determined. However, potential explanations for our findings include exercise-induced changes of hormones, nutritional changes, doping or a genetic predisposition. Acute exercise has been shown to temporarily elevate DHEA and other androgens, although these return to baseline values within hours (24). IGF-I levels have been observed to increase, decrease or show no change in response to acute and chronic exercise (110, 121, 177, 178, 180, 199, 200). Although the anabolic hormonal changes due to exercise are not completely clarified, we performed the blood sampling in a rested state, thereby excluding the possible acute exercise changes in hormone levels. Additionally, even though IGF-I levels are known to increase in response to amino acids/protein, whereas IGFBP-1 decrease, acute nutritional effects are not likely to have influenced our results, since all blood samples were collected in a fasted state. On the other hand, chronic energy deficiency reduces IGF-I levels (107, 201) and increases IGFBP-1 (109, 144, 201). Although this condition is common among elite athletes (126), the finding of higher IGF-I levels in the athletes compared to controls argues against chronic energy deficiency having an impact on our results.

Androgen supplementation affects urine and especially serum androgen levels in women (61). Anabolic agents such as AAS, GH and IGF-I are used as doping agents among elite athletes (7, 166, 171). We acknowledge that we did not evaluate the potential misuse of GH or IGF-I

in our study population. However, all female athletes that participated in the studies are regularly included in doping control tests. The urinary steroid profile of endogenous AAS and synthetic AAS were analyzed for all participants and none demonstrated atypical findings. Therefore, we believe that doping was not the reason for higher precursor androgens and IGF-I and IGFBP-1 in the athletes.

Speculatively, our observed findings of higher precursor androgens and IGF-I and IGFBP-1 in the female athletes compared to controls may reflect a genetic predisposition for a more anabolic hormonal constitution. For IGBP-1 and particularly IGF-I concentrations there is a strong genetic influence, determining 36% and 63% of the variance, respectively (202). Furthermore in athletes, endogenous IGF-I levels have been shown to be stable with low intra-individual variability (14-16%) (203). The heritability of T and SHBG have also been estimated to be high. In post-menopausal sisters and twins, the heritability of T and SHBG was 39% and 56 %, respectively (204). Another study including women >19 y, not using exogenous hormones or being pregnant and with intact ovaries, found that total, free T and SHBG showed moderately to high heritability estimates (25-56%) after adjusting for age, BMI, diabetes, current smoking and menopausal status (205). Furthermore, in a subset of female twins not using hormonal contraceptives, included in the follicular phase of the menstrual cycle, the T level heritability estimate was 51% (206). Additionally, PCOS a common condition among female elite athletes, is suggested to have a genetic association (136, 138, 142, 207). PCOS is also a known cause for menstrual dysfunction among female Olympic athletes and athletes with PCOS have demonstrated both higher T and DHEA-S compared to regularly menstruating elite athletes (136). PCOS was not evaluated in our study population, however as reported in study I, menstrual dysfunction was more common among female athletes than controls and the athletes demonstrated higher precursor androgen levels. Considering this, there is the possibility of some of the athletes included in this study having PCOS.

7.1.1 Anabolic hormones depending on sport category

Although we hypothesized higher androgens levels among power athletes compared to endurance and technical athletes no significant differences in androgen levels were observed between groups. In a large study by Bermon et al (142), it was reported that T and DHEA-S were higher in athletes participating in throwing and jumping events compared to long distance events. Furthermore, athletes participating in strength, explosive and power sports have been found to have higher precursor androgens compared to long distance runners (19). In our population of power athletes, age-adjusted IGF-I levels were highest compared to the other sport categories. In agreement, higher serum IGF-I levels have been observed in gymnast

compared to runners (176). However, in a large study by Healy et al (14), swimmers and cross-country skiers were reported to have the highest IGF-I levels. We chose to divide the athletes into sport categories of power, endurance, technical disciplines with reference to a previously published study by Hagmar et al (136). However, the discrepancy between our findings and previous reports may be due to the heterogeneity of included sports in each sport category. Furthermore, the number of participants in each sport category was limited and our results should therefore be interpreted with caution.

7.2 ENDOGENOUS ANABOLIC HORMONES, BODY COMPOSITION AND PHYSICAL PERFORMANCE

7.2.1 Body composition

The existing literature on the relationship between endogenous anabolic hormones, such as androgens and IGF-I and body composition in female elite athletes is very limited. Several factors are suggested to regulate body composition in female athletes such as exercise, nutrition and anabolic hormones (4, 12, 208). For example, endogenous androgens promote bone formation, have anabolic effects in muscle tissue and stimulate the erythropoiesis (4, 149-151), whereas IGF-I stimulates protein synthesis in human skeletal muscle and has anabolic effects on adult bone remodeling (107, 108). These effects may be beneficial for athletic performance and possibly reduce the risk of injury in elite athletes. In this thesis, we report new findings on the relationship between endogenous anabolic hormones, body composition and physical performance in elite female athletes.

Not surprisingly, we found that the female athletes had significantly higher BMD and lean mass and lower body fat percent compared to untrained female controls. There is substantial evidence that athletes demonstrate higher BMD and lean mass compared to sedentary controls (138, 209-211). In agreement with our results with power athletes demonstrating the highest BMD, high impact sports such as volleyball and soccer are associated with higher BMD compared to low impact sports such as swimming (212). Increased mechanical loading leads to structural adaptations that stimulate bone formation and improve bone strength (207). The intense training regimes undertaken by athletes may therefore explain the differences in body composition observed between athletes and controls (208, 210, 212). Furthermore, exercise generally promotes an increase in muscle mass and a decrease in fat mass i.e. leanness (high muscle/lean mass, low fat mass), having a positive influence on physical performance in many

sports (213). However, it is highly likely that these adaptations to exercise are driven by the effects of androgens and other anabolic hormones such as IGF-I.

In support of an anabolic role of endogenous androgens for BMD and lean mass in female athletes, we found significant positive correlations between precursor androgens, T and BMD as well as between DHEA, 5-DIOL and Etio-G and lean mass. These findings remained in the subgroup of athletes not using hormonal contraceptives. Comparable results have been observed in postmenopausal women (158, 159), in non-athletic PCOS women (145) and in a small cohort of female athletes (138). Furthermore, in the total population of athletes and controls, we found that endogenous IGF-I and IGFBP-1 were positively associated with BMD and lean body mass. Others have reported a positive correlation between IGF-I and body weight (177), BMD and lean mass (176). However, most previous assumptions on IGF-I anabolic effects on body composition in athletes are extrapolated from studies investigating exogenous GH and IGF-I in GH-deficient adults (164, 167). In contrast, in recreational athletes IGF-I supplementation resulted in no significant change in body composition (173). Interestingly, in muscle cells, a locally produced IGF-I isoform (MGF) is expressed as a result of mechanical stimuli. This IGF-I isoform increases with exercise and is believed to be important for satellite cell activations and muscle regeneration (121). The role of circulating vs. locally produced IGF-I was not evaluated in this thesis, however theoretically both higher circulating IGF-I and locally produced IGF-I, the latter as a result higher training load in the athletes, may result in positive anabolic effects on muscle tissue.

7.2.2 Physical performance

In support of a role of endogenous anabolic hormones for physical performance in female Olympic athletes we observed significantly positive associations between DHEA, 5-DIOL and DHT with SJ and CMJ performance. Multiple regression analyses showed that DHEA was the strongest factor predicting SJ, whereas lean mass was the most significant determinant for CMJ. Previous investigations on the relationship between endogenous androgens and physical performance have demonstrated a positive association between serum T and CMJ in 22 female athletes (16). Furthermore, in a large observational study by Bermon et al (19) female athletes with the highest tertile of free T levels had a competitive advantage in certain sport events (400 m, 400 m hurdles, 800 m, hammer throw, pole vault) compared to female athletes in the lower free T tertile.

We found no significant correlations between serum T and physical performance in our cohort of female Olympic athletes. However, in women, intracellular transformation of DHEA

accounts for approximately 50 % of the T production and DHEA is therefore suggested to be the most important precursor androgen in women (26, 214). This is in contrast to men where the majority of endogenous T production takes place in the testis, whereas very small amounts are produced from DHEA (148). In male athletes, DHEA supplementation has not demonstrated any anabolic effects (215). It is possible that the same relationship is true for women with supra physiological T levels, whereas for women with endogenous T levels within the normal range (such as our study cohort), DHEA may be more important for athletic performance (216). This is supported by our findings of significant positive correlations between DHEA and physical performance. This hypothesis is further strengthened by muscle biopsy studies in non-athletic women, where adding DHEA resulted in significantly increased intramuscular T but not estradiol. Furthermore, intramuscular estradiol, T, DHT and DHEA were independent predictors of muscle strength and power (217).

Further supporting a significant role of endogenous androgens for increased muscle mass and athletic performance is the 15-times higher endogenous T levels and the superior physical performance of men compared to women and pre-pubertal boys and girls (218). Since exogenous T and endogenous T have identical chemical structures and similar biological effects (4), studies evaluating the effects of T supplementation are also of interest. In men, T supplementation results in a dose-dependent increase in muscle mass and strength (4, 153, 219) and there is a growing body of evidence for similar findings in postmenopausal women (156) and premenopausal healthy, trained, women (18). Further solidifying the positive anabolic effects of androgens in athletes, is the use of AAS with reports of increased strength and training ability (7). Investigations on the use of AAS in female athletes is limited, however documentation of the experiments performed in the DDR, revealed that athletes were subjected to AAS from an early age and that the performance enhancing effects were especially prominent in women (15). In addition, there is an overrepresentation of hyperandrogenic conditions such as PCOS (136, 220-223) and 46 XY DSD (142) among female athletes compared to the general population. Moreover, for women with DSD a reduction of T levels resulted in a 5-7 % decrease in physical performance (147). Interesting data is also emerging from studies investigating the effects of altered anabolic hormonal levels on muscle volume and physical performance in transgender men and women. In transgender men (female to male transgender), T supplementation, resulting in increased T levels in adult male range, significantly increased both muscle mass, hemoglobin levels and grip strength (224, 225), whereas in transgender women (male to female) a reduction in T levels from adult male to female range reduced muscle mass by 9.4 % and hemoglobin levels by 14 % (224). More recently, Wiik and colleagues (157) observed a robust increase in muscle volume and muscle

strength, the latter by approximately 12 % following 12 months of cross-hormone treatment in transgender men. In transgender women a slight decrease in muscle mass was evident, whereas muscle strength was maintained.

An alternative explanation for the sex difference in athletic performance between men and women are anabolic effects of endogenous GH and IGF-I. GH secretion is however higher in women compared to men (4) whereas IGF-I is comparable between sexes (111). To our knowledge no previous studies have investigated the role of endogenous IGF-I for physical performance in female elite athletes. However, in untrained women, IGFBP-2 has been associated with peak oxygen uptake (174). In contrast, in adolescent girls, IGF-I was not related to physical fitness measured by aerobic performance (175). In our population of athletes, we did observe significant positive correlation between IGF-I and IGFBP-1 and SJ and squat performance, respectively. These results may support an anabolic role of IGF-I in female athletes however the correlations were weak.

The majority of evidence for the positive anabolic effects of GH originates from studies investigating GH and IGF-I supplementation to GH deficient adults (164, 166, 167), whereas comparable findings in healthy subjects and elite athletes have been proven difficult to reproduce (166, 168, 169, 226). The exception is the study by Meinhardt et al (172) where GH supplementation was given to recreational athletes resulting in a slight improvement in sprint performance in men but not in women. These effects were significantly amplified in male participants receiving T supplementation in addition to GH. Androgens and estrogens are suggested to be connected to GH/IGF secretion. Androgens have been shown to increase GH and IGF-I secretion following aromatization to estrogen (114, 115), and estrogen supplementation depending on distribution route can increase or decrease serum IGF-I levels (116). In elderly women, DHEA supplementation increases IGF-I levels (227). However, this has not yet been replicated in recreational female athletes, possibly due to a small study sample or short durations of supplementation (228).

Taking both previously published findings and the results presented in this thesis, we suggest that endogenous androgens, as well as endogenous IGF-I play a role for increased muscle mass and athletic performance in female athletes.

7.3 THE 2D:4D RATIO IN FEMALE ELITE ATHLETES

In the largest study to date including only top-elite female athletes, we found that the 2D:4D ratio right hand was lower in the female Olympic athletes compared to the control group, suggesting a higher prenatal androgen exposure in the athletes. No significant difference was observed in the left 2D:4D ratio. This is in line with previous work primarily in male athletes (97, 98, 100), and it has been suggested that the right 2D:4D ratio is more representative of prenatal androgen exposure (79). In agreement with our results, previous studies investigating the digit ratio in female athletes at national level (99), college tennis players (100), youth handball players (98) and non-elite athletes (97) have found a lower 2D:4D in the athletes compared to controls.

In addition, we found significant correlations between the 2D:4D ratio and increased strength and faster 3000 m running times in the athletes. The few previous studies that have investigated the digit ratio and athletic performance in female athletes, have found faster skiing times (103), faster rowing times (102), greater running performance (229) and better national fencing level (101) to be related to a lower 2D:4D ratio. Taken together, this supports a role of prenatal androgen exposure for physical performance in female elite athletes.

The explanation for the association between the 2D:4D ratio and athletic ability is not elucidated. Several plausible mechanisms have been proposed such as a possible connection between the 2D:4D ratio and adult serum androgen levels, genetic variants in the AR-receptor and other genes coding for androgen metabolizing enzymes (80, 104). There is increasing evidence of the positive effects of endogenous and exogenous androgens on physical performance in women and female athletes (8, 16, 18, 136, 182). However, in agreement with our findings in study II, recent studies have found no robust evidence for an association between the 2D:4D ratio and adult serum androgens (104, 105). The role of genetic variations in the AR-receptor in relation to the 2D:4D ratio have also been investigated, with the conclusion that the digit ratio is not associated with the AR-receptor (104, 106). Polymorphism in other genes coding for enzymes important for T metabolism and function, such as CYP19A1 (aromatase), SRD5A2 (5α-reductase) and SHBG genes (SHBG), have recently been evaluated showing no evident associations with the 2D:4D ratio (104). Even so, studies have indicated that the 2D:4D ratio is a heritable trait and that the 2D:4D ratio may be associated with other genetic factors (230-233).

Interestingly, we were first to demonstrate that the 2D:4D ratio was negatively correlated to certain urinary steroid glucuronide metabolites (T-G, 5αAdiol-17G, 5βAdiol-17G). As previously described, the conjugation of androgens by androgen metabolizing enzymes (phase II enzymes i.e., UGTs and SULTs) represents the final step in the androgen metabolism. Androgens are then mainly excreted in urine (21, 22, 47, 48). The urinary androgen levels are dependent on the activity and expression of UGTs and SULTs and there are known polymorphisms in the genes coding for these enzymes associated with adult urinary androgen levels (21, 45, 181). Several fetal UGTs are expressed in the first trimester (234), it is therefore possible that genetic polymorphism in UGTs may be associated with the androgen load of the fetus during the first trimester, the time of pregnancy when the 2D:4D ratio is set (77, 78). Speculatively, genetic variations in genes coding for phase II enzymes could perhaps influence the development of the 2D:4D ratio and the predisposition for physical performance later in life and future studies could investigate this in a larger group.

7.4 THE URINARY STEROID PROFILE IN FEMALE ELITE ATHELTES

In female athletes, the interpretation of doping tests is complex possibly due to the impact of hormonal contraceptive use, hormonal fluctuations during the menstrual cycle, genetic variations affecting steroid metabolizing enzymes and exercise. It is therefore important to evaluate the effects of these factors on the urinary steroid profile.

7.4.1 The impact of hormonal contraceptives and genetic variations on the urinary steroid profile

We found that hormonal contraceptive use and genetic polymorphisms in UGT2B17 and CYP17 had an impact on the urinary steroid profile in female athletes. More specifically individuals with a deletion polymorphism in UGT2B17 excreted significantly lower T-G and had a lower T/E ratio compared to ins/del and ins/ins individuals. This is in line with previously published findings in men (21, 45). In an investigation including only four women, hormonal contraceptive use was found to affect the T/E ratio and suppression of EpiT levels were suggested as the mechanism behind these findings (68, 71). We could confirm this in a larger sample of female athletes (n=79), as we observed that female athletes using hormonal contraceptives had significantly lower EpiT-G levels and subsequently a higher T/E ratio. We noted similar findings in the separate populations of female Olympic athletes (n=94) and controls (n=86) included in study V. In a recently published interventional study the urinary steroid profile was examined in 55 regularly menstruating women, prior to and after three

months of using a COC containing levonorgestrel and ethinylestradiol (235).The authors found a decline in all urinary steroids included in the ABP although the largest decrease was observed for EpiT levels. Consequently, the T/E ratio increased. Taking this into account we considered the impact of hormonal contraceptives on the EpiT-G levels in women to be quite robust although it is possible that different hormonal contraceptives and COC types and the duration of treatment may result in varied EpiT responses.

The production and metabolism of EpiT is largely unknown, although it has been suggested that EpiT is formed from pregnenolone by CYP17A1 (Figure 4). As mentioned in section 3.2.1.1, a promotor region polymorphism (T>C exchange) in the CYP17A1 gene has been described. The CC variant is hypothesized to upregulate gene expression, and in men this variant has been associated with higher excretion of EpiT-G (45, 52). In female athletes using hormonal contraceptives we found similar results, with the CYP17A1 C-allele carriers having significantly higher EpiT-G and T-G levels compared to the TT genotype. In the previously mentioned intervention study by Ekström et al (235), it was noted that the HC induced changes in EpiT were associated with the CYP17A1 genotype, with individuals with a T-allele demonstrating a larger suppression of EpiT compared to individuals with a CC genotype. Among athletes not using hormonal contraceptives, we found no significant difference in EpiT levels depending on CYP17A1 genotype. A possible explanation and confounding factor being that in women not using hormonal contraceptives, EpiT levels seem to vary across the menstrual cycle with higher levels at ovulation and the luteal phase and higher T/E and 5αAdiol/E ratios in the follicular phase (70, 188). Whereas in women using hormonal contraceptives, EpiT levels varied less (71). If urine samples had been collected in the same cycle phase for all participants, it is possible that the difference in EpiT levels depending CYP17A1 genotype may have been evident for hormonal contraceptive users as well.

7.4.2 The urinary steroid profile in female athletes and controls

To our knowledge, this is the first time a difference in urinary steroid profile between female athletes and sedentary controls has been demonstrated, with significantly lower urinary steroid metabolite levels observed in the athlete population. This difference was confirmed using both GC-MSMS (study V) and LC-MSMS (unpublished data). Similar findings have been described when comparing the urinary steroid metabolites between male athletes and controls (236). In the subgroup of athletes and controls not using hormonal contraceptives, comparable results were found. The lower urinary steroid levels observed in the athletes does not reflect the serum androgen levels. Indeed, no significant differences were observed between groups in T or DHT

and the precursor androgens DHEA and 5-DIOL were actually higher in the total population of female Olympic athletes compared to the control group.

In women, the excretion of T is significantly higher in competition compared to out of competition and stress has been proposed as an influencing factor that may increase the excretion rate of urinary steroids (237). In our population of athletes and controls there were no significant differences in cortisol levels (a hormone that increases during stress). On the other hand, in the subgroup not using hormonal contraceptives, athletes actually had significantly higher cortisol levels compared to controls. Therefore, the influence of stress cannot likely explain the lower urinary steroid metabolites in the athletes. Other factors that may contribute to altered steroid profiles in women is genetic variations in especially UGT2B17. However, since the frequency of UGT2B17 del/del individuals were comparable between groups this should not have influenced our findings.

A hypothetical explanation may be that androgens in athletes are also eliminated by additional routes e.g. in feces and/or sweat. In female athletes there are no studies investigating the excretion of steroids in feces. However, T and DHT were found to be highly abundant in feces from eight healthy men (44). Due to the lipophilic properties of steroids, sweat might also be a potential excretion route (43). It has been shown that steroid metabolites included in the ABP (A and T) can be excreted as sulphate conjugates in human axillary sweats (238). Additionally, after transdermal application of estr-4-ene diol, the metabolites nortestosterone and estr-4-enedione were found in sweat collected after physical exercise (42). Therefore, one might speculate that the negative correlations between amount of training (hours per week) and certain urinary steroid metabolite concentrations may be due to alternative excretions routes such as sweat. However, further studies are needed to understand the association between training and urinary excretion rate of androgens in athletes.

7.5 STRENGHTS AND LIMITATIONS

In this thesis, we present novel findings on the associations between endogenous anabolic hormones, the 2D:4D ratio, body composition and physical performance in female Olympic athletes. We also provide results on the impact of hormonal contraceptives and genetic variations on the urinary steroid profile in a larger number of top female elite athletes. Additionally, we demonstrate for the first time, lower urinary androgen metabolite concentrations in female elite athletes compared to untrained controls.

The results presented in this thesis are of importance for the ongoing discussion regarding hyperandrogenism in women athletes. In fact, the results from study I were part of the research presented to and evaluated by CAS, leading to updated regulations concerning hyperandrogenism in women athletes in 2018. In addition, our results on the impact of hormonal contraceptives and genetics on the urinary steroid profile in female athletes may contribute to improving doping interpretation.

Professor Angelica Lindén Hirschberg and associate professor Bo Berglund responsible for the research project are physicians at the SOC. Therefore, we were able to recruit such a unique study group i.e. female Olympic athletes. The recruitment was challenging due to the busy training and travelling schedule of the athletes and in the end, this led to recruitment taking several years (2011-2015). Some of the athletes were recruited at training camps, making it difficult for them to participate in the DXA examination and standardized physical performance tests at Bosön in Stockholm. Even so, we were able to recruit a large number of female Olympic athletes.

In an effort to overcome potential confounding factors such as nutrition and effects of acute exercise/stress, that could theoretically influence circulating androgens, IGF-I and the urinary steroid profile blood and urine samples were collected in the morning in a rested and fasted state, for all participants. An additional strength of this thesis was that we analyzed the complete serum androgen profile by the golden standard method LC-MS/MS and the urinary steroid profile was determined by WADA accredited methods, GC-MS and GC-MS/MS at the accredited anti-doping Laboratory, Karolinska University Hospital, Huddinge.

However, certain limitations should be addressed. Due to the cross-sectional study design casualty cannot be determined. Still, we believe that observational studies within this field can provide important knowledge, especially since the researcher is limited to the type of studies that can be performed in elite athletes. For example, supplementation of anabolic hormones would be highly unethical since these compounds are classified as doping agents.

We were not able to take into account hormonal contraceptive use as well as the phase of the menstrual cycle as this would have been too challenging and result in fewer athletes being interested in participating in the studies. It has previously been reported that approximately 47 % of female Swedish Olympic athletes use hormonal contraceptives (136). Furthermore, menstrual dysfunction is common among female elite athletes (134-136). Therefore, to only include female athletes not using hormonal contraceptives and with regular menstruation would

have resulted in significant selection bias and a reduced sample size, increasing the risk of type II error.

Hormonal contraceptive use is known to affect serum T and SHBG levels (30, 139), whereas the impact on serum precursor androgens is less established (30, 140, 141). The influence of hormonal contraceptives on IGF-I levels is not fully elucidated. Estrogen therapies are suggested to alter the GH/IGF axis in women, the effects are dependent on type of estrogen, route of administration, duration of treatment and dose (116). However, the frequency of hormonal contraceptive use was comparable in our population of athletes and controls. Additionally, there were no statistically significant interactions between hormonal contraceptive use and endogenous androgens and IGF-I when comparing groups. When adding hormonal contraceptive use to the regression model in study I, we found no significant interaction and the reported correlations between lean mass and physical performance in the whole population of athletes were similar among athletes not using hormonal contraceptives. Also, in study III there were no significant differences in IGF-I or IGFSD depending on hormonal contraceptive use. Furthermore, in study V the differences in urinary steroid levels between athletes and controls were similar in the subgroup not using hormonal contraceptives. In the light of this, one can assume that the differences observed between groups and the significant correlations were not dependent on hormonal contraceptive use.

Considering menstrual cycle phase, there is a small mid-cycle increase in T whereas previous investigations have found no significant variance in DHT, 5-DIOL DHEA and DHEAS levels across the menstrual cycle (4, 128-131). The variations in IGF-I levels during the menstrual cycle have been described to be none (119) or modest (117, 118) whereas unchanged levels have been reported for IGFBP-1 (117, 119). Therefore, even though blood samples were collected randomly during the menstrual cycle we do believe that the results presented are valid. Urinary androgen metabolite levels, especially EpiT fluctuates during the menstrual cycle (70). However, since doping tests are collected randomly, the results presented in study IV and V can provide valuable information.

Athletes were divided into subgroups: power, endurance and technical as previously described (136). We do acknowledge the heterogeneity of sports included and that the limited number of athletes in each subgroup may have limited the ability to detect differences between sport categories.

During the process of writing study III, an unintentional error was discovered regarding the height and weight of the athletes compared to controls. In study I, table 1, age, BMI, height and

weight of the athletes and controls were switched. The correct results are presented in this thesis and in study III. All original data and published findings were examined thoroughly, and no additional errors were found. Importantly, our main results were not affected by this mistake.

In conclusion, even though certain limitations are evident we believe that the results presented in this thesis are valid and provide important new knowledge to the field of sports endocrinology.

8 CONCLUSION AND FUTURE DIRECTIONS

8.1 CONCLUSION

In conclusion, we observed higher precursor androgens and IGF-I levels in female Olympic athletes than in controls suggesting a more anabolic endocrine profile in the athletes. Furthermore, the lower 2D:4D ratio in the athletes compared to controls indicates a higher androgen exposure during fetal life in the athletes. Serum androgens and IGF-I levels were related to body composition, whereas both these anabolic hormones as well as the 2D:4D ratio were related to physical performance tests in the athletes. Furthermore, in female athletes, hormonal contraceptive use and genetic polymorphism in UGT2B17 and CYP17 had an impact on the urinary Epi-T levels as well as the T/E ratio. Urinary androgen metabolite levels were significantly lower in female athletes compared to untrained controls and additionally, the metabolite levels were negatively associated with training hours per week in the athletes.

8.1.1 Study specific conclusions

I The higher levels of endogenous precursor androgens (within the normal range) observed in female athletes compared to controls, and the significant correlations between androgens, lean mass and physical performance in the athletes, support a role of endogenous androgens in athletic performance in female athletes.

II The lower 2D:4D ratio in female athletes than controls, and the association between a low 2D:4D ratio and physical performance tests in the athletes, suggest that a higher prenatal androgen exposure may be of importance for athletic capacity in female Olympic athletes.

III The higher endogenous IGF-I and IGFBP-1 levels observed in the athletes and the association with increased lean mass, lower fat percent and better SJ and squats performance may suggest that IGF-I and IGFBP-1 have an impact on body composition and physical performance in female athletes.

IV Hormonal contraceptive use and UGT2B17 and CYP 17 polymorphisms affect the urinary steroid profile significantly, which should be considered in the evaluation of doping tests.

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V Urinary androgen metabolites monitored in the ABP are lower in the female athletes than in untrained controls and negatively related to training hours per week in the athletes. However, serum androgen levels were comparable between groups. This may indicate a higher androgen metabolite excretion by alternative routes, such as feces and sweat in the female athletes.

8.2 FUTURE DIRECTIONS

This thesis sheds some light on the role of endogenous anabolic hormones in physical performance in female athletes and factors that have an impact on the urinary steroid profile in women.

We found significant correlations between endogenous serum anabolic hormones, lean mass and physical performance. To further elucidate how circulating hormones affect physical performance in female athletes, it would be of great interest to study intracellular processes, such as the metabolism of DHEA and locally produced isoforms of IGF-I, in muscle tissue, an important organ in regard to explosive and strength performance.

In the context of anti-doping testing, the quantification of endogenous serum steroids may be implemented as a complementary approach to the urinary steroid profile method in the future. Therefore, it would be of interest to study serum androgens and androgen metabolites longitudinally to evaluate intra-individual stability and the impact of different types of hormonal contraceptives and exercise in female elite athletes.

We were intrigued by the unexpected finding of lower androgen metabolite levels in the female athletes compared to controls. We plan to investigate this further by studying different excretion routes after acute and chronic training in athletes and controls.

It would also be of great interest to study the dose-response effect of exogenous anabolic hormones on physical performance. However, supplementation of T and/or GH/IGF-I cannot be performed in elite athletes but is theoretically possible in recreational female athletes under controlled conditions regarding duration and dose of treatment.

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